Effects of Distinct Populations of Adenosine Receptors in the Ventral Striatum on Cocaine Seeking

by

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A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado in partial fulfillment
of the requirement for the degree of
Doctor of Philosophy
Department of Psychology and Neuroscience
2015

This thesis entitled:
Effects of Distinct Populations of Adenosine Receptors in the Ventral Striatum on Cocaine Seeking
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Thesis directed by Assistant Professor Ryan K. Bachtell, Ph.D.

Abstract

Drug-associated cues or pharmacological stimuli induce cocaine seeking by enhancing dopamine and glutamate neurotransmission in the nucleus accumbens (NAc). Adenosine is an inhibitory neuromodulator of dopamine and glutamate signaling and represents a viable target for decreasing relapse vulnerability. Postsynaptic adenosine A_1 receptors and adenosine A_{2A} receptors co-localize with dopamine receptors on distinct populations of medium spiny neurons in the NAc. The co-localization of dopamine and adenosine receptors is meaningful in that adenosine receptor stimulation antagonizes dopamine receptor signaling and alters the activity of NAc output pathways. Presynaptic adenosine receptors are expressed on glutamate terminals in the NAc where A_1 receptors inhibit and A_{2A} receptors enhance glutamate release in the NAc. The overarching goal of these studies was to determine the how distinct populations of adenosine receptors modulate striatal signaling to influence cocaine seeking. Our results indicate that adenosine receptors oppositely modulate cocaine seeking depending on the receptor subtype and their synaptic locale. Postsynaptic adenosine A_{2A} receptor stimulation in the NAc decreases cocaine seeking by disrupting dopamine D_2 receptor signaling in the NAc. Postsynaptic blockade of adenosine A_{2A} receptors enhances cocaine seeking by facilitating dopamine D_2 receptor signaling. Blockade of

presynaptic adenosine A_{2A} receptors, on the other hand, reduces cocaine seeking, potentially by tempering augmented glutamate release that drives reinstatement. Lastly, adenosine A_1 receptor stimulation or presynaptic adenosine A_{2A} receptor blockade during extinction produces long-term changes in relapse susceptibility. The findings suggest that modulating specific populations of NAc adenosine receptors are influential in cocaine seeking and may represent viable pharmacotherapeutic strategies.

Dedication

This work is dedicated to my grandparents: Tom Myers, Judy Myers, Joann O'Neill, and Pete O'Neill.

Acknowledgements

I am deeply thankful to have had Dr. Ryan Bachtell as a mentor in this program. His guidance has been integral to any successes I have had as a graduate student. I am grateful for everything he has taught me, for his patience with my bad ideas (and my propensity for crying), and his belief in me as a scientist. I'm so happy to have been a part of building this lab as his first graduate student. His work ethic is unmatched and his enthusiasm (even in the face of reviewers' inane comments) has instilled in me the importance of hard work and tenacity. In addition to being one of the most hardworking scientists I know, he is also one of the most fun. Few advisors truly care about the happiness of their students, but with Ryan's support I'm able to leave this program with nostalgia for all the great times I've had in Boulder and, even, with a minimal amount of sanity.

I'd also like to thank the numerous undergraduates and techs that have helped me to complete the work in this thesis as well as countless other projects. I am forever in debt to our truly amazing PRAs including Sophia Levis, Drew Schriener, McKenzie LeTendre, Kevin Kavanagh, and, our most recent addition, Maddi Winkler. I've been particularly pleased to mentor and receive the benefits of so many undergraduates in my time here including Ben Hobson, Jake Stafford, Talia Scott, Sarah Cross, Kayla Brown, Hollene Hongdoxmai, and Solana Archuleta.

I owe everything that I am to my parents Dianne and Patrick O'Neill. Their belief in me has seen me through every hardship and has taught me to pursue my goals in the face of any obstacles. My maternal grandparents Judy and Tom Myers and my paternal

grandmother Joann O'Neill have also contributed to my successes, and I will forever be grateful to have been born into such a supportive and loving family. Nothing seems impossible with people like this on your side.

Special thanks also go to my fiancé, Ryan Newsom. He has seen me at my very worst and somehow still finds me tolerable. His support throughout this program and his willingness to adventure the American West with me has made my years in graduate school some of the best of my life. We've put in countless hours in the lab, seeing music, on mountain tops, and in the yoga studio and there's no one I'd rather be on this journey with.

Finally I'd like to thank my friends here in graduate school for their willingness to hang out with me, listen to me whine, and their unwavering encouragement through the rough times. Thank you so much Rachel Daut, Mike Weber, Erika Galer, Rob Rozeske, Zach Smith, Florencia Bercum, Amanda Ellis, Austin Hudmon, Laura Fonken, Mike Barrata, and Bryce Skarda you guys are the greatest.

“No more time to tell how
This is the season of what
Now is the time of returning
With thought jewels polished and gleaming

Now is the time past believing
The child has relinquished the reign
Now is the test of the boomerang
Tossed in the night of redeeming

Eight sided whispering hallelujah hatrack
Seven faced marble eye transitory dream doll
six proud walkers on jinglebell rainbow
Five men writing in fingers of gold
Four men tracking down the great white sperm whale
Three girls wait in a foreign dominion

Ride in the whale belly
Fade away in moonlight
Sink beneath the waters
to the coral sand below”

-Robert Hunter

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Chapter 1: Introduction and Overview

History and Significance

Cocaine is a naturally occurring alkaloid found in the leaves of the *Erythroxylon coca* plant. Cocaine makes up only 1% of the coca leaf when grown in high altitudes, and people living in the Andes mountains have chewed these leaves for at least 1200 years (Altman *et al*, 1985). Ancient civilizations in South America, Mexico, Indonesia and the West Indies used coca leaves for medicinal, religious and ceremonial reasons. The arrival of the Spanish in South America eventually brought coca to Europe where the cocaine alkaloid was first isolated in the mid-1800s by a German PhD student. It became a popular additive to wines, tonics, and patent medicines, and was even marketed as a cure for morphine addiction in the late 1800s (Goldstein *et al*, 2009). However, by the turn of the century cocaine's addictive properties and potential for serious medical complications became apparent and in 1914 it became illegal in the United States. By the 1920s cocaine use had significantly declined partially due to the introduction of amphetamine (Dackis and O'Brien, 2001). It wasn't until the 1960s that cocaine resurfaced as a popular drug of abuse with a glamorous reputation (Goldstein *et al*, 2009). By the late 1970s epidemic levels of cocaine addiction developed in the United States, this was especially spurred by the advent of "crack," an affordable free-base form of cocaine (Dackis *et al*, 2001).

Cocaine abuse in the United States persists as a significant public health problem. The National Survey on Drug Use and Health estimates that more than 34

million Americans over the age of 12 have used cocaine at least once in their lifetime, and 2.1 million are current users of cocaine (SAMHSA, 2008). Cocaine is the most common drug-related cause of emergency department visits in the United States (NIDA, 2009). More than 1.3 million people in the United States needed treatment for cocaine addiction, but less than 300,000 were actually admitted in to treatment facilities in 2008 (SAMHSA, 2008). Unfortunately, rates of relapse following treatment for addiction are high, between 40-60% (NIDA, 2009). Not only does addiction impact the addicted individual's life negatively, frequently leading to incarceration, job loss and homelessness, its negative consequences reach beyond the individual resulting in higher crime rates and approximately 181 billion dollars in economic cost to the United States every year (NIDA, 2009).

The Nature of Cocaine Addiction

Addiction can be described as cyclic pattern of use, abstinence, and relapse. Initial drug use is voluntary and activates the reward pathways of the brain resulting in euphoria in the user. These positive reinforcing effects of the drug are what primarily drive continued use in the beginning stages of addiction. However, chronic cocaine use produces neuroadaptations and users frequently experience withdrawal symptoms (Cornish and Kalivas, 2001; Dackis *et al*, 2001; Leshner and Koob, 1999; Thomas *et al*, 2008). These adaptations mediate the transition from use motivated by the positive reinforcing effects of cocaine to use motivated by the negative reinforcing effects of cocaine, like decreasing unpleasant withdrawal symptoms. Much research has

suggested that repeated cocaine use decreases behavioral control, and addicts in this stage of addiction find themselves going to extraordinary lengths to acquire cocaine (Cornish *et al*, 2001; Dackis *et al*, 2001; Leshner *et al*, 1999; Thomas *et al*, 2008; Volkow and Fowler, 2000; Volkow *et al*, 1992). At this stage, many addicts will attempt to quit using. Unfortunately, because cocaine addiction involves the primary reward centers of the brain it takes on the characteristics of a primary survival drive, thus maintaining abstinence difficult and relapse common (Dackis *et al*, 2001; Leshner *et al*, 1999).

Criteria put forth by the American Psychiatric Association (2013) for diagnosing substance use disorder are divided into 4 clusters: impaired control (e.g. inability to decrease drug use), social impairment (e.g. failure to fulfill major obligations due to drug use), risky use (e.g. recurrent use in hazardous situations), and pharmacologic dependence (e.g. withdrawal symptoms when not using or using less). Cocaine addiction is characterized by compulsive drug use despite negative consequences, and high rates of relapse even after long periods of abstinence. Continued cocaine seeking is driven by the desire to feel the euphoria or “high” associated with administration coupled with the need to suppress the negative symptoms (e.g. anhedonia and anxiety) that accompany withdrawal from chronic use (Kiyatkin, 1994; Leshner *et al*, 1999). Cocaine craving and relapse to drug taking in abstinent human addicts is typically precipitated by one of three major stimuli: a stressful life event, a stimulus previously associated with drug use, or reexposure to cocaine itself (Leshner *et al*, 1999; Schmidt and Pierce, 2010; Shaham *et al*, 2003).

Intravenous Drug Self-Administration and Reinstatement

The scientific community has developed several models of cocaine addiction. Intravenous (IV) self-administration of cocaine, in which animals perform an operant task (e.g. lever-pressing) to self-administer a drug via an IV catheter, is the most common animal model of relapse (Kalivas and McFarland, 2003; Schmidt *et al*, 2010). To model relapse, rodents typically undergo a period of cocaine self-administration followed by extinction of the drug-reinforced behavior (e.g. previously drug-paired lever pressing) in the absence of cocaine. Once animals have adequately extinguished, the ability for stress, drug-associated stimuli, or cocaine to reinstate non-reinforced lever pressing is assessed. This model of relapse has proved to have excellent face validity in that stimuli that trigger cocaine craving in human addicts also produce reinstatement in animals (Epstein *et al*, 2006). The predictive validity of this model, however, is unclear since we have yet to uncover a successful pharmacotheraphy to prevent relapse to cocaine addiction (Epstein *et al*, 2006). Despite this drawback, the reinstatement model of addiction has good construct validity that has proven useful in elucidating the cellular and molecular mechanisms as well as the neural circuitry underlying cocaine seeking behavior (Kalivas *et al*, 2003; Nestler, 2005; Volkow *et al*, 2000).

Mechanisms of Cocaine Action in the Dopamine System

Cocaine blocks the reuptake of all the monoamines dopamine, norepinephrine, and serotonin although neuropharmacological studies have established a critical role for

central nervous system dopamine in the acute rewarding effects of cocaine. Mice lacking expression of the dopamine transporter (DAT) gene failed to show increased locomotor activity to cocaine (Giros *et al*, 1996), and showed dramatically decreased self-administration of cocaine compared to serotonin transporter (SERT) knockout mice (Thomsen *et al*, 2009). Additionally, mice engineered to express a cocaine-insensitive DAT show decreased cocaine reward in cocaine conditioned place preference and self-administration paradigms (Chen *et al*, 2006; Thomsen *et al*, 2009). Low doses of dopamine receptor antagonists reliably block cocaine conditioned place preference (Pruitt *et al*, 1995), development and expression of cocaine sensitization (Fontana et al, 1993), and acquisition of cocaine self-administration (De Wit and Wise, 1977; Leshner *et al*, 1999; Woolverton, 1986; Woolverton and Johnson, 1992).

There are two primary dopamine systems within the brain. The nigrostriatal system projects from the substantia nigra to the caudate putamen, and the mesocorticolimbic dopamine system that projects from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), frontal cortex and amygdala (Thomas *et al*, 2008). The nigrostriatal system is typically associated with movement generation for well-established behavior patterns. The mesocorticolimbic system, on the other hand, has been primarily implicated in goal-directed learning and motivated behavioral outcomes. Thus, this system has been associated with the reinforcing actions of cocaine based on a number of studies. First, this pathway way is necessary for the reinforcing effects of cocaine since selective destruction of dopamine neurons in the mesocorticolimbic pathway with 6-hydroxydopamine abolished cocaine self-administration (Roberts *et al*,

1980). Second, increased extracellular dopamine in the NAc is a common characteristic of acute responses to addictive substances, including cocaine (Di Chiara, 1995; Di Chiara and Imperato, 1988; Pontieri *et al*, 1995).

Neurons originating in the VTA release dopamine into terminal areas such as the NAc upon presentation of salient stimuli and during episodes of reward-based learning. The NAc consists (~90%) of medium spiny GABA projection neurons (MSNs) and a variety of inhibitory (GABA-expressing) and excitatory (acetylcholine-expressing) interneurons. Of these, the MSNs have received the greatest attention since they are the primary projection neurons and form two distinct output pathways (Aubert *et al*, 2000; Steiner and Gerfen, 1998). The direct pathway MSNs express the opioid peptide dynorphin and project back to the VTA, while the indirect pathway MSNs express the opioid peptide enkephalin and innervate the ventral pallidum, a key output structure of the ventral striatum (Cornish *et al*, 2001; Lu *et al*, 1998). In addition to the direct and indirect pathways being distinguished by opioid peptide expression they also display distinct expression of the two main subtypes of dopamine receptors, dopamine D_1 and dopamine D_2 receptors (Lu *et al*, 1998). The direct pathway expresses dopamine D_1 receptors and the indirect pathway expresses dopamine D_2 receptors (see figure 1.1). There is a subset of MSNs that express both dopamine D_1 and D_2 receptors, however these neurons make up less than 10% of the neurons within the NAc and the functional significance and neuroanatomical projection sites are less clear (Girault, 2012).

Dopamine D_1 and D_2 receptors are distinguished based on their G-protein coupling and the downstream effects on cAMP production and PKA-mediated signaling

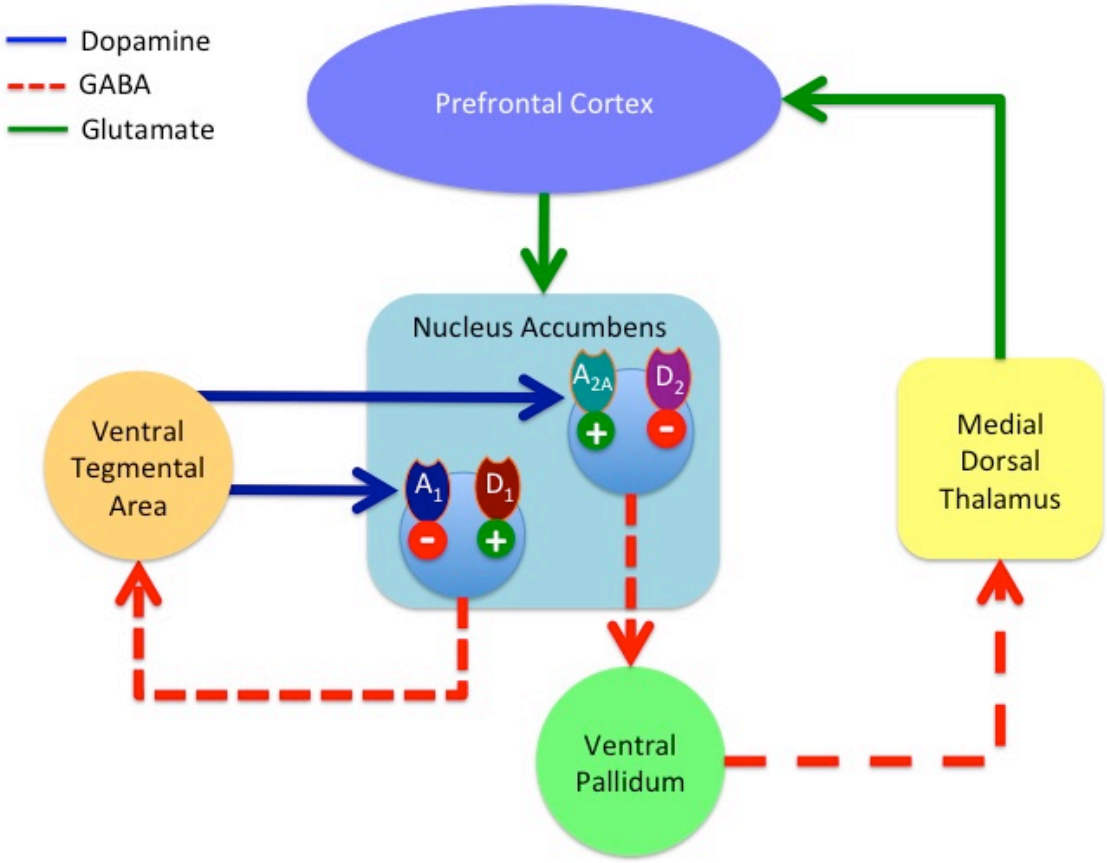

Figure 1.1 Direct and Indirect Pathways of the Ventral Striatum. Dopamine from the ventral tegmental area stimulates dopamine receptors on medium spiny GABA neurons in the nucleus accumbens. Dopamine D_1 receptors are expressed on the direct pathway which projects to the ventral tegmental area, and dopamine D_2 receptors are expressed on the indirect pathway which projects to the ventral pallidum.

(Lachowicz and Sibley, 1997). Dopamine D_1 receptors signal through $G\alpha_{s/olf}$ to stimulate adenylyl cylclase, leading to the production of cAMP, and the activation of PKA (Sibley *et al*, 1998). In contrast, dopamine D_2 receptors signal through $G\alpha_{i/o}$ inhibiting adenylyl cyclase, decreasing production of cAMP, and limiting PKA activation (Lachowicz *et al*, 1997). DARPP-32, a phosphoprotein regulated by cAMP and dopamine, is a major target of PKA and is highly expressed in dopamine responsive striatal and cortical neurons (Borgkvist and Fisone, 2007; Svenningsson *et al*, 2005). DARPP-32 integrates signals from multiple neurotransmitters to bidirectionally modulate PKA activity, and plays a critical role in the regulation of downstream signal transduction pathways (Greengard *et al*, 1999). Dopamine D_1 receptor stimulation increases PKA phosphorylation of DARPP-32 which then inhibits PP1, an inhibitor of PKA, to further enhance PKA activation (Greengard *et al*, 1999). Conversely, dopamine D_2 receptor stimulation results in dephosphorylation of PP2B, and this converts DARPP-32 into a potent inhibitor of PKA (Borgkvist *et al*, 2007; Svenningsson *et al*, 2005). Interestingly, the loss DARPP-32 in dopamine D_1 neurons decreases cocaine-induced locomotion, while loss of DARPP-32 in dopamine D_2 neurons increases cocaine-induced locomotion (Bateup *et al*, 2010).

Postsynaptic dopamine receptor stimulation also modulates the direct and indirect pathway neurons by influencing glutamate input to the ventral striatum. Studies have indicated that activation of the PKA cascade through dopamine D_1 receptor stimulation has direct effects on the trafficking and function of inonotropic glutamate receptors. Dopamine D_1 receptor activation of PKA increases surface expression of

GluA1 containing α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and *N*-methyl-D-aspartate (NMDA) receptors (Hallett *et al*, 2006; Snyder *et al*, 2000), and also enhances NMDA receptor activated currents in these neurons (Cepeda *et al*, 1993; Liu *et al*, 2004). Dopamine D_1 receptor stimulation has also been shown to alter Ca^{2+} and K^+ channels to enhance glutamate signaling (Gerfen and Surmeier, 2011; Lobo and Nestler, 2011; Surmeier *et al*, 2007). Conversely, dopamine D_2 receptor stimulation decreases the activity of PKA to inhibit the excitability of the indirect pathway. In fact, dopamine D_2 receptor stimulation promotes the dephosphorylation of serine 845 residue on the GluA1 subunit to decrease surface expression of AMPA receptors (Hakansson *et al*, 2006), and decreases AMPA receptor currents in indirect pathway neurons (Cepeda *et al*, 1993). Dopamine D_2 receptor stimulation also alters Ca^{2+}, Na^+, and K^+ channels to decrease glutamate signaling (Gerfen *et al*, 2011; Lobo *et al*, 2011; Surmeier *et al*, 2007). Thus, dopamine input to the NAc modulates excitatory glutamate input onto medium spiny neurons to ultimately determine behavioral output.

Both types of dopamine receptors play an important role in mediating the addictive properties of psychostimulants like cocaine. It appears that dopamine D_1 receptors are more important for the initial rewarding/stimulating effects of cocaine, but that dopamine D_2 receptors may ultimately become more important in mediating the effects of chronic cocaine use. Studies in non-cocaine addicted humans have also shown that the subjective effects of methylphenidate, a drug that mimics the mechanism of cocaine, are correlated with dopamine D_2 receptor occupancy (Volkow *et al*, 1999b). In this study, lower levels of dopamine D_2 receptor occupancy were correlated with

increased “liking” (Volkow *et al*, 1999b), but in chronic cocaine users cue-induced craving was positively correlated with dopamine D_2 receptor occupancy (Wong *et al*, 2006). This idea is further supported by the fact that antagonism of dopamine D_1 receptors block the acquisition and expression of cocaine conditioned place preference, but dopamine D_2 receptor antagonism has no effect (Cervo and Samanin, 1995). In locomotor sensitization models of addiction, dopamine D_1 and D_2 receptors are necessary for the development of cocaine sensitization, but only dopamine D_2 receptors are necessary for its expression (Fontana *et al*, 1993).

Additionally, PET studies of human cocaine addicts show decreases in dopamine D_2 receptor expression, but no change in dopamine D_1 receptors (Martinez *et al*, 2004; Volkow *et al*, 1993; Volkow *et al*, 1990). Interestingly, chronic cocaine self-administration in rats has been shown to increase the expression of dopamine D_2 receptors existing in high affinity state, which may explain why a decrease is observed in human addicts (Briand *et al*, 2008). However, in self-administration models both dopamine D_1 and D_2 antagonists administered in the nucleus accumbens decrease cocaine self-administration (Barrett *et al*, 2004), but another study found that after 2 week access to cocaine dopamine D_1 receptor antagonism had less pronounced effects on progressive ratio responding of animals exposed to long access cocaine self-administration sessions (Ramoa *et al*, 2014). In reinstatement paradigms systemic administration of dopamine D_2 receptor, but not dopamine D_1 receptor agonists, induces cocaine seeking (Self *et al*, 1996). In fact, systemic administration of dopamine D_1 receptor agonists or antagonists block cocaine-primed reinstatement (Khroyan *et al*,

2000). It is important to note, however, that stimulation of both dopamine D_1 and D_2 receptors in the NAc is sufficient to produce reinstatement responding (Bachtell *et al*, 2008; Schmidt and Pierce, 2006b).

Neuroadaptations in Glutamate Transmission

Glutamate is the main excitatory neurotransmitter in the central nervous system, and it is estimated that more that 80% of the synapses in the brain use glutamate (Siegel et al, 2006). For many years research focused on the contribution of dopamine to addictive processes, but in the past 15 years glutamate has emerged as an increasingly important neurotransmitter in relapse and reinstatement of cocaine seeking behavior. Glutamate input to the NAc (see figure 1.1) comes from several sources including the medial prefrontal cortex (mPFC), amygdala, and hippocampus (Britt et al, 2012; Carlezon and Thomas, 2009). While each of these brain regions can regulate certain aspects of reinstatement responding, numerous studies have revealed that glutamate signaling from mPFC to the NAc is necessary to induce cocaine seeking (Cornish et al, 1999; Cornish and Kalivas, 2000; Kalivas, 2004; McFarland and Kalivas, 2001; McFarland et al, 2003). Extracellular glutamate levels in the nucleus accumbens are largely unaffected by acute cocaine administration, but after chronic cocaine taking and withdrawal animals show disrupted glutamate homeostasis (Kalivas, 2009). This disrupted glutamate homeostasis is defined by decreased basal levels of extracellular glutamate, but exacerbated increases in extracellular glutamate in the NAc in response to a cocaine challenge (Kalivas, 2009). Studies have established that the decreases in

basal glutamate levels observed after withdrawal from chronic cocaine self-administration are a result of reduced activity of the cystine glutamate-antiporter (Baker *et al*, 2003a; Baker *et al*, 2003b).

Glutamate signaling is mediated by several subfamilies of ionotropic receptors including: NMDA receptors and AMPA receptors. These tetrameric receptors are made up of different subunit compositions that provide functional diversity among the subfamilies. The contribution of NMDA receptors to cocaine addiction is unclear, conflicting reports have been published indicating both decreases and increases of NR1 and NR2A/B receptor subunits following withdrawal from cocaine-self administration (Gass and Olive, 2008; Lu *et al*, 2003; Self *et al*, 2004), and intra-accumbens administration of both NMDA receptor agonists and antagonists produce reinstatement in rats (Cornish *et al*, 1999; Cornish and Kalivas, 2000; Famous *et al*, 2007). AMPA receptors, on the other hand, show increases in GluA1 subunit surface expression following chronic cocaine self-administration (Conrad *et al*, 2008). Interestingly, re-exposure to cocaine has been shown reduce surface expression of GluA1 subunits 24 hrs later (Boudreau *et al*, 2007). However, increases in phosphorylation of GluA1 subunits immediately following dopamine D_1 receptor stimulation appear to mediate cocaine seeking (Anderson *et al*, 2008; Hobson *et al*, 2013).

Glutamate signaling is also mediated by metabotropic glutamate receptors (mGluRs) that modulate intracellular signaling pathways through their associated G proteins. There are three main families of mGluRs: group I (mGluR 1 and 5), group II (mGluR 2 and 3), and group III (mGluR 4 and 6-8). Systemic or intra-NAc blockade of

mGluR5 decreases cocaine-primed reinstatement (Kumaresan *et al*, 2009; Lee *et al*, 2005). Stimulation of mGluR2/3 has been shown to decrease cocaine-primed reinstatement, however these effects were also seen on food seeking indicating possible non-specific effects (Peters and Kalivas, 2006). Although it is not fully clear how the receptors that mediate glutamate transmission are regulated following chronic cocaine self-administration, the necessity of glutamate release from the mPFC to the accumbens in cocaine-induced drug seeking has been established.

Following cocaine self-administration and extinction a systemic injection of cocaine produces a robust increase in glutamate in the NAc, which can be attenuated by inactivation of the mPFC (McFarland *et al*, 2003) or by AMPA receptor blockade in the NAc (Cornish *et al*, 2000). Similarly, cocaine administered directly into the mPFC induces reinstatement that can be blocked by antagonism of AMPA receptors in the NAc (Park *et al*, 2002). Cocaine-primed reinstatement is also associated with decreased extracellular GABA in the ventral pallidum, presumably arising from indirect pathway neurons in the accumbens. Thus, this decreased GABA input to the ventral pallidum is dependent on glutamate release from the mPFC to the NAc (Tang *et al*, 2005; Torregrossa *et al*, 2008). Taken together these finding strongly support the idea that activation of glutamatergic projections from the mPFC to the NAc are responsible for cocaine seeking, and this is further substantiated by human studies demonstrating that cocaine craving in addicts is accompanied by increased mPFC activation (Volkow *et al*, 1999a; Volkow *et al*, 2005).

Past and Present Pharmacotherapies for Cocaine Addiction

There are currently no approved pharmacotherapies for cocaine addiction. In the United States, treatment for cocaine addiction ranges from residential in-patient treatment facilities to intensive outpatient programs, cognitive behavioral therapy, individual psychotherapy, family therapy, and self-help groups. Typically treatment focuses on detoxification, behavioral modifications, and strategies to cope with craving. While non-pharmacological therapies are likely affecting brain function, the ultimate solution to decreasing relapse susceptibility may be the discovery of pharmacotherapies that can normalize the neuroadaptations produced by chronic cocaine use.

Since the 1980s numerous treatments for cocaine addiction have been assessed, and relatively few have shown any significant effects. Because cocaine reward heavily relies on increased dopamine signaling antipsychotics, therapeutics that mainly block dopamine receptors, were some of the earliest treatments for cocaine dependence. A meta-analysis of 10 studies found that antipsychotics did not differ from placebo in decreasing the number of cocaine use days, severity of addiction, or cocaine craving (Kishi *et al*, 2013). Unfortunately, in addition to having no effect on cocaine dependence, antipsychotics also produced severe side effects (e.g. depression, dizziness, akathisia) leading to greater rates of intolerability-related discontinuation (Kishi *et al*, 2013). Dopamine agonists have also been assessed as a potential substitution-based pharmacotherapy for cocaine addiction. Although the side effects were less severe, no improvement in abstinence from cocaine was seen (Amato *et al*, 2011). Due to cocaine's effects on serotonin reuptake, antidepressants have been

examined for effectiveness in producing abstinence from cocaine. Similarly, a meta-analysis of these studies found no significant effects on abstinence from cocaine compared to placebo (Pani *et al*, 2011).

More recently, interest in glutamate mechanisms of cocaine addiction led to the development of d-cycloserine as a therapeutic for treatment of cocaine dependence. D-cycloserine is a partial agonist at NMDA receptors, and has been shown to facilitate extinction of cocaine self-administration in rodents (Thanos *et al*, 2011a; Thanos *et al*, 2011b). However, in a randomized, placebo-controlled study of human addicts, d-cycloserine did not facilitate extinction of cocaine craving, but may have enhanced it (Price *et al*, 2013). No differences were observed between groups in post-extinction cocaine use (Price *et al*, 2013).

The most recently developed pharmacotherapy for cocaine addiction is *N*-acetylcysteine, a prodrug that drives the cystine-glutamate antiporter. *N*-acetylcysteine shows promise as an antirelapse medication. As previously mentioned, disruption of the cystine-glutamate antiporter is responsible for the decreased levels of basal glutamate in the nucleus accumbens following chronic cocaine self-administration (Baker *et al*, 2003a; Baker *et al*, 2002). Administration of *N*-acetylcysteine restores basal glutamate levels and normalizes corticostriatal function (Moussawi *et al*, 2011). *N*-acetylcysteine administered prior to extinction training or during abstinence prevents reinstatement to cocaine seeking for two weeks after administration by reversal of the neuroplasticity required for reinstatement (Madayag *et al*, 2007; Moussawi *et al*, 2011; Reichel *et al*, 2011). Preliminary studies in human cocaine addicts show decreased cocaine craving to

an experimenter delivered IV injection of cocaine following 4 days of *N*-acetylcysteine administration (Amen *et al*, 2011), decreased responsiveness to cocaine cues while taking *N*-acetylcysteine, and facilitates termination/reduction of cocaine use in treatment seeking individuals (Mardikian *et al*, 2007). In a larger double-blind placebo-controlled trial *N*-acetylcysteine failed to decrease cocaine use in cocaine-dependent volunteers, but significantly decreased craving and time to relapse in subjects who had already achieved abstinence before entering the trial (LaRowe *et al*, 2007).

Adenosine: A Neuromodulator of Dopamine and Glutamate Signaling

Adenosine is a neuromodulator of both dopamine and glutamate signaling and for this reason, among others, it has become a viable target for decreasing relapse vulnerability (Fuxe *et al*, 2007a). Adenosine is tonically released into the synapse through bidirectional nucleoside transporters. Phasic release occurs through the vesicular release and subsequent metabolism of adenosine triphosphate (ATP) in the synapse (Fredholm and Dunwiddie, 1988; Fredholm *et al*, 1984; Svenningsson *et al*, 1999a). Adenosine signals through two main receptor subtypes expressed in the central nervous system, adenosine A_1 and A_{2A} receptors (Ferre, 1997; Ferre *et al*, 1992). Adenosine A_1 receptors are widely expressed in the brain, existing presynaptically on most glutamate and dopamine terminals and postsynaptically on cortical and hippocampal pyramidal neurons (Fuxe *et al*, 2007a; Linden, 1991). Importantly, adenosine A_1 receptors are also expressed postsynaptically on MSNs in the striatum and are highly colocalized with dopamine D_1 receptors in the direct pathway (see figure

1.1) (Aubert *et al*, 2000; Fuxe *et al*, 2007a). Adenosine A_{2A} receptors, on the other hand, have dense expression in the striatum, but little expression in other brain regions (Svenningsson *et al*, 1999b). Adenosine A_{2A} receptors are typically postsynaptic on MSNs in the striatum and are highly colocalized with dopamine D_2 receptors in the indirect pathway (see figure 1.1) (Ferre *et al*, 1993a; Svenningsson *et al*, 1997). Presynaptic adenosine A_{2A} receptors exist in smaller proportions and are localized to a subset of glutamate terminals that specifically synapse onto direct pathway MSNs (Quiroz *et al*, 2009; Rosin *et al*, 2003).

Both types of adenosine receptors are G protein coupled with adenosine A_1 receptors being inhibitory ($G\alpha_{i/o}$) and adenosine A_{2A} receptors being stimulatory ($G\alpha_{s/olf}$) (Ferre *et al*, 1997; Linden, 1991; Svenningsson *et al*, 1999b). Importantly, the G protein coupling of adenosine A_1 and A_{2A} receptors opposes the intracellular signaling cascades of dopamine D_1 and D_2 receptors, respectively, on MSNs where these receptors are colocalized (Ferre *et al*, 1997). In addition to their opposing intracellular signaling effects, adenosine and dopamine receptors form heteromeric receptor complexes (A_1-D_1 and A_{2A}-D_2) where adenosine and dopamine receptors have direct antagonistic interaction with one another (Ferre *et al*, 2004; Fuxe *et al*, 2008).

In fact, stimulation of adenosine A_1 receptors results in decreased binding affinity at dopamine D_1 receptors and facilitates the formation of the A_1-D_1 heteromeric receptor complex (Ferre *et al*, 1994b; Ferre *et al*, 1998; Fuxe *et al*, 2007a), while dopamine D_1 receptor stimulation decreases the expression of the A_1-D_1 receptor heteromers (Franco *et al*, 2003). Although most of this work has been done in vitro, co-immunoprecipitation

experiments have verified the existence of this heteromer in the rat striatum and have shown that repeated cocaine injections disrupt the expression of the A_1-D_1 heteromer (Toda *et al*, 2003). Interestingly, receptors maintain their heteromeric receptor complexes with co-administration of both an adenosine A_1 receptor agonist and a dopamine D_1 receptor agonist, but show decreased dopamine D_1 signaling to adenylyl cyclase indicating a possible uncoupling of the $G\alpha_{s/olf}$ from the dopamine D_1 receptor (Gines *et al*, 2000). In the absence of the A_1-D_1 heteromeric complex the antagonistic interactions of the receptors play out through their intracellular signaling cascades. Thus, the presence of adenosine A_1 receptor agonists leads to a decrease in dopamine D_1-induced cAMP production, and the presence of adenosine A_1 receptor antagonists potentiate the production of cAMP in response to dopamine D_1 receptor stimulation (Ferre *et al*, 1998).

In the indirect pathway MSNs, adenosine A_{2A} receptor stimulation decreases the binding affinity of dopamine D_2 receptors likely due to a conformational change in the binding pocket (Salim *et al*, 2000). Additionally, adenosine A_{2A} receptor activation leads to a reduction of $G\alpha_{i/o}$ coupling to the dopamine D_2 receptor (Ferre *et al*, 1993b). Adenosine A_{2A} receptor stimulation has also been shown to counteract dopamine D_2 receptor-induced intracellular calcium responses (Salim *et al*, 2000). Stimulation of dopamine D_2 receptors decreases firing rates of the indirect pathway MSNs, and this effect can be enhanced by antagonism of adenosine A_{2A} receptors and attenuated by adenosine A_{2A} agonists (Stromberg *et al*, 2000). Thus, in experimental conditions adenosine A_{2A} receptor agonists function similarly to dopamine D_2 receptor antagonists,

and removal of the adenosine A_{2A} receptors' tonic inhibition on dopamine D_2 receptors results in decreased activation of the indirect pathway. As with adenosine and dopamine heteromeric receptor complexes in the direct pathway, cocaine decreases the expression of A_{2A}-D_2 heteromers in the indirect pathway (Marcellino *et al*, 2010). With regard to the opposing intracellular cascades of adenosine A_{2A} and dopamine D_2 receptors, dopamine D_2 receptors can inhibit adenosine A_{2A} receptor-induced increases in cAMP (Ferre *et al*, 1993a; Fuxe *et al*, 2005; Kull *et al*, 1999; Svenningsson *et al*, 1999a). Additionally, activation of adenosine A_{2A} receptors facilitates increased excitability in the indirect pathway MSNs through amplified PKA activity that results in greater phosphorylation of AMPA and NMDA receptors and results in increased phosphorylation of DARPP-32, a target potently inhibited by dopamine D_2 receptor agonists (Fuxe *et al*, 2007a; Fuxe *et al*, 2007b; Hakansson *et al*, 2006; Hakansson *et al*, 2004). In microdialysis experiments, intra-NAc administration of an adenosine A_{2A} receptor agonist increases extracellular levels of GABA in the ventral pallidum (Ferre *et al*, 1994a), where decreases in GABA are necessary for reinstatement of cocaine seeking (Tang *et al*, 2005; Torregrossa *et al*, 2008).

Presynaptic expression of adenosine A_1 and A_{2A} on glutamate terminals in the nucleus accumbens provide an important means to regulate phasic glutamate release (Orru *et al*, 2011b). Here stimulation of adenosine A_1 receptors inhibits the release of glutamate, while stimulation of adenosine A_{2A} receptors facilitates glutamate release (Ciruela *et al*, 2006; Orru *et al*, 2011b). On these glutamate terminals adenosine A_1 and A_{2A} receptors have also been shown to form heteromeric receptor complexes (Ciruela *et*

al, 2006; Quiroz *et al*, 2009). These heteromers appear to be a functional "molecular switch" that controls glutamate release in response to extracellular levels of adenosine (Ciruela *et al*, 2006). Consequently, adenosine A_1 and A_{2A} receptors have opposing effects on intracellular signaling, and low concentrations of adenosine promote signaling through adenosine A_1 receptors inhibiting glutamate release (Ciruela *et al*, 2006; Ferre *et al*, 2008; Quiroz *et al*, 2009). Under conditions of high extracellular adenosine, glutamate release is stimulated by signaling through the adenosine A_{2A} receptor, which inhibits adenosine A_1 receptor signaling through intramembrane receptor interaction (Ciruela *et al*, 2006; Ferre *et al*, 2007; Ferre *et al*, 2008; Quiroz *et al*, 2009). It is unclear how chronic cocaine administration effects extracellular adenosine levels, but repeated cocaine has been shown to increase adenosine tone in the VTA (Bonci and Williams, 1996).

Due to the ability of adenosine receptors to modulate activity of dopamine and glutamate in the striatum, adenosine receptors are practical targets for tempering the neuroadaptations seen after chronic cocaine use. In fact, several studies have indicated a role for adenosine receptors in cocaine-mediated behaviors. Increased in locomotor activity to acute administration of cocaine is reduced by the adenosine A_{2A} receptor agonist, CGS 21680, and enhanced by the adenosine A_{2A} receptor antagonist, MSX-3, (Poleszak and Malec, 2002b; Rimondini *et al*, 1997). Systemic A_{2A} receptor stimulation also impairs the initiation of cocaine self-administration (Knapp *et al*, 2001) and reduces cocaine sensitization (Filip *et al*, 2006). Non-selective antagonism of adenosine A_1 and A_{2A} receptors induces reinstatement (Green and Schenk, 2002; Weerts and Griffiths,

2003), and stimulation of either adenosine A_1 or A_{2A} receptors blocks the expression of cocaine sensitization (Hobson *et al*, 2012) and attenuates cocaine seeking (Bachtell and Self, 2009; Hobson *et al*, 2013; Weerts *et al*, 2003). Additionally, blockade of adenosine A_{2A} receptors reverses reward impairments produced by cocaine withdrawal (Baldo *et al*, 1999). Based on these findings, adenosine receptors appear to have the ability to modulate multiple cocaine-related behaviors through their effects on dopamine and glutamate transmission. The work presented here will focus on elucidating the specific role of several populations of adenosine receptors in cocaine seeking, and present a novel target for preventing relapse.

Chapter 2: Adenosine A_{2A} Receptors in the Nucleus Accumbens Bi-directionally Alter Cocaine Seeking in Rats

Abstract

Repeated cocaine administration enhances dopamine D_2 receptor sensitivity in the mesolimbic dopamine system, which contributes to drug relapse. Adenosine A_{2A} receptors are colocalized with D_2 receptors on NAc medium spiny neurons where they antagonize D_2 receptor activity. Thus, A_{2A} receptors represent a target for reducing enhanced D_2 receptor sensitivity that contributes to cocaine relapse. The aim of these studies were to determine the effects of adenosine A_{2A} receptor modulation in the NAc on cocaine seeking in rats that were trained to lever press for cocaine. Following at least 15 daily self-administration sessions and 1 week of abstinence, lever pressing was extinguished in daily extinction sessions. We subsequently assessed the effects of intra-NAc core microinjections of the A_{2A} receptor agonist, CGS 21680 (4-[2-[[6-amino-9-(*N*-ethyl-*b*--ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride), and the A2A receptor antagonist, MSX-3 (3,7-dihydro-8-[(1*E*)-2-(3-methoxyphenyl)ethenyl]-7-methyl-3-[3-(phosphonooxy)propyl-1-(2-propynyl)-1*H*-purine-2,6-dione disodium salt hydrate), in modulating cocaine- and quinpirole-induced reinstatement to cocaine seeking. Intra-NAc pretreatment of CGS 21680 reduced both cocaine- and quinpirole-induced reinstatement. These effects were specific to cocaine reinstatement as intra-NAc CGS 21680 had no effect on sucrose seeking in rats trained to self-administer sucrose pellets. Intra-NAc treatment with MSX-3 modestly reinstated

cocaine seeking when given alone, and exacerbated both cocaine- and quinpirole-induced reinstatement. Interestingly, the exacerbation of cocaine seeking produced by MSX-3 was only observed at sub-threshold doses of cocaine and quinpirole, suggesting that removing tonic A_{2A} receptor activity enables behaviors mediated by dopamine receptors. Taken together, these findings suggest that A_{2A} receptor stimulation reduces, while A_{2A} blockade amplifies, D_2 receptor signaling in the NAc that mediates cocaine relapse.

Introduction

The mesolimbic dopamine (DA) system is involved in many aspects of addiction, including drug reward, craving, and relapse behaviors (Shaham *et al*, 2003; Shalev *et al*, 2002). Activation of this pathway through stress exposure, drug associated cues, and pharmacological stimuli is known to mediate relapse to cocaine seeking (Shaham *et al*, 2003). The mesolimbic DA system consists of DA cells in the VTA that project to the NAc among other forebrain targets.

Drugs of abuse stimulate DA release in the NAc that is mediated by two major classes of DA receptors that are distinguished by their intracellular signaling cascades among other aspects. DA binding at dopamine D_1 receptors increases adenylyl cyclase activity, while DA binding at dopamine D_2 receptors decreases the activity of this enzyme (Lachowicz *et al*, 1997). In addition, dopamine D_1 and D_2 receptors are primarily expressed on two distinct populations of NAc neurons, with dopamine D_1 receptors occurring mainly on dynorphin/substance P-expressing neurons and dopamine D_2 receptors on enkephalin-expressing neurons (Lu *et al*, 1998). These subpopulations of neurons comprise the direct and indirect striatal pathways, respectively, that differ in their projection targets as well as their influence on behavioral output (Aubert *et al*, 2000; Steiner *et al*, 1998).

Repeated cocaine administration produces alterations in DA receptor-mediated responses. Thus, repeated cocaine administration produces cross-sensitization with dopamine D_2 receptor agonists (Ujike *et al*, 1990), and while dopamine D_1 and D_2 receptors are necessary for the acquisition of behavioral sensitization, only dopamine

D_2 receptors are necessary for its expression (Fontana *et al*, 1993). In self-administration models, systemic and intra-accumbens stimulation of dopamine D_2 receptors produces robust reinstatement to cocaine seeking (Bachtell *et al*, 2005; De Vries *et al*, 1999; Dias *et al*, 2004; Khroyan *et al*, 2000; Schmidt *et al*, 2006b; Self *et al*, 1996), and dopamine D_2 receptors appear to mediate cue-induced relapse to cocaine seeking (Cervo *et al*, 2003; Gal and Gyertyan, 2006). Therefore, tempering dopamine D_2 receptor-mediated behaviors following chronic cocaine administration could prove useful in preventing relapse.

A known modulator of DA neurotransmission is adenosine. Adenosine activity is mediated by subtypes of adenosine receptors including adenosine A_{2A} receptors that are heavily expressed in the striatum, where they are highly co-localized with dopamine D_2 receptors on enkephalin-containing neurons of the indirect pathway (Fink *et al*, 1992; Svenningsson *et al*, 1999b). Adenosine A_{2A} receptors exert tonic inhibitory control over dopamine D_2 receptor signaling within the striatum (Farrar *et al*, 2010; Hakansson *et al*, 2006; Harper *et al*, 2006; Nagel *et al*, 2003; Weber *et al*, 2010). Thus, adenosine A_{2A} receptor stimulation decreases DA binding at dopamine D_2 receptors (Ferre *et al*, 1991b). A recent study has suggested that this may be mediated by heteromeric receptor complexes comprised of adenosine A_{2A} and dopamine D_2 receptors (Marcellino *et al*, 2010). Interestingly, cocaine was shown to reduce the expression of the A_{2A}-D_2 receptor heteromer, which may partially explain the enhanced dopamine D_2 receptor-mediated behaviors following repeated cocaine administration (Marcellino *et al*, 2010).

Recent studies have shown an involvement of adenosine A_{2A} receptors in the behavioral effects of cocaine. For example, systemic adenosine A_{2A} receptor stimulation impairs the initiation of cocaine self-administration (Knapp *et al*, 2001), reduces cocaine sensitization (Filip *et al*, 2006), and blocks reinstatement of cocaine seeking (Bachtell *et al*, 2009). The non-specific adenosine antagonist, caffeine, produces modest reinstatement (Green *et al*, 2002; Worley *et al*, 1994), while specific antagonism of adenosine A_{2A} receptors enhances cocaine sensitization (Filip *et al*, 2006). It remains unclear whether these adenosine A_{2A} receptor effects on cocaine behaviors are mediated by adenosine A_{2A} receptors in the NAc. Therefore, the present study examines whether adenosine receptor effects on cocaine seeking are mediated by adenosine A_{2A} receptors localized to the NAc. These experiments test the effects of intra-NAc adenosine A_{2A} receptor stimulation or blockade on cocaine seeking in animals extinguished from cocaine self-administration. Local infusions of CGS 21680, a selective adenosine A_{2A} receptor agonist, and MSX-3, a phosphatase prodrug of the adenosine A_{2A} receptor antagonist MSX-2 (Muller *et al*, 1998; Sauer *et al*, 2000), were made into the medial division of the NAc core, a site where dopamine D_2 receptor stimulation is sufficient for reinstatement (Bachtell *et al*, 2005; McFarland and Kalivas, 2001).

Materials and Methods

Animals

Male Sprague-Dawley rats (Charles River, Wilmington, MA) initially weighing 275-325 grams were individually housed with food and water available ad libitum. All experiments were conducted during the light period of a 12-hr light/dark cycle in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the University of Colorado at Boulder.

Surgery

Surgical implantation of jugular catheters and intracranial cannulae occurred in concert. Catheters were implanted into the jugular vein under halothane anesthesia (1-2.5%). Each rat was then placed into a stereotaxic instrument, the scalp was incised and retracted, and the head was positioned with Bregma and Lambda at the same depth coordinate. Screws were secured into the skull and holes were drilled in order to bilaterally insert guide cannulae into the NAc core (A/P: +1.7, M/L +/-1.5, D/V -5.7 from bregma; (Paxinos and Watson, 1998). Once inserted, the guide cannulae were fixed in place with dental cement. Dummy stylets extending 1 mm beyond the tip of the cannulae were placed into the guide cannulae to maintain patency. Animals showing signs of post-surgical distress were administered (S)-(+)-Ketoprofen (5mg/kg), a nonsteroidal anti-inflammatory analgesic (Carabaza *et al*, 1996). Catheters were flushed daily with 0.1 mL heparinized saline and rats were allowed 4-7 days recovery in their home cage before experimental procedures began.

Drugs

Adenosine A_{2A} receptor agonist, CGS 21680 [4-[2-[[6-Amino-9-(N-ethyl-b-D-ribofuranuronamidosyl)-9H -purin-2-yl]amino] ethyl]benzenepropanoic acid hydrochloride] was purchased from Tocris Bioscience (Ellisville, MO). Adenosine A_{2A} receptor antagonist, MSX-3 [3,7-dihydro-8-[(1E)-2-(3- methoxyphenyl)ethenyl]-7-methyl-3-[3- (phosphonooxy)propyl-1-(2-propynyl)-1H-purine-2,6-dione disodium salt hydrate], dopamine D_2-selective agonist, Quinpirole [(-)-Quinpirole hydrochloride], and Cocaine hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO). All drugs were dissolved in sterile-filtered physiological (0.9%) saline.

Cocaine self-administration, extinction and reinstatement procedures

Self-administration procedures were performed in operant conditioning chambers (Med-Associates, St. Albans, VT) equipped with two response levers and an infusion pump system. Animals were initially trained to lever press for sucrose pellets to facilitate acquisition of cocaine self-administration. After 24-48 hr of food-restriction, rats were trained to lever-press for sucrose pellets on a fixed ratio 1 (FR1) reinforcement schedule until acquisition criteria was achieved (100 sucrose pellets in one session). After lever-press training, animals were fed ad libitum for at least 1 day prior to surgery (see above).

After recovery from surgery, animals were allowed to self-administer intravenous cocaine (0.5 mg/kg/100 µL injection) on a fixed ratio 1 (FR1) reinforcement schedule in daily 4-hr sessions for 5–6 d/wk. Cocaine injections were delivered over 5 s concurrent with the illumination of a cue light above the active lever and was followed by a 15 s

time out period (TO 20s) when the house light remained off and responding produced no consequence. Inactive lever responses produced no consequence throughout testing.

After a minimum of 15 cocaine self-administration sessions, animals remained in their home cages for 7 days of forced abstinence. On days 8-13 following self-administration, animals returned to the operant conditioning chambers for extinction training. Extinction sessions occurred in the absence of cocaine reinforcement in 4-hr test sessions. Responses on the lever previously paired with cocaine injections during self-administration (drug-paired lever) and on the inactive lever were recorded but had no programmed drug or cue delivery.

Each reinstatement session was initiated with 2-hr of extinction conditions followed by a 2-hr reinstatement test period. In most experiments, an intra-NAc pretreatment was administered prior to a pharmacological prime (see below), which was immediately followed by the 2-hr reinstatement test period. Responses at both the previously drug-paired and inactive levers were recorded but resulted in no cue or drug delivery during testing.

A_{2A} antagonist (MSX-3)-primed reinstatement

Two groups of animals were used to assess the effects of systemic and intra-NAc treatments of MSX-3 on reinstatement. MSX-3 is a prodrug of the selective adenosine A_{2A} receptor antagonist MSX-2 that is rapidly converted to its active form by phosphatases in vivo (Muller *et al*, 1998; Sauer *et al*, 2000), and has been shown to be

suitable for intracranial microinfusion (Hauber *et al*, 1998). Animals in one group were given systemic injections of MSX-3 (vehicle, 3, and 6 mg/kg, i.p.) following the extinction session. Animals in a separate group were given intra-NAc injections of MSX-3 (vehicle, 5, 10, and 20 ug/side). Immediately following the systemic treatments and 5 min after the intra-NAc microinjections the animals underwent 2-hr of reinstatement testing. Animals in both groups were tested under all conditions in a randomized order and received a maximum of 4 treatments. Responses at both levers were recorded, but resulted in no cue or cocaine delivery.

Effects of A_{2A} receptor stimulation and blockade on cocaine-primed reinstatement

The effects of intra-NAc adenosine A_{2A} receptor stimulation on cocaine-primed reinstatement were tested by a pretreatment of the adenosine A_{2A} agonist, CGS 21680 (vehicle, 0.5, 1.0, 2.5, 5.0 and 10 ng/side), 5 min prior to the priming injection of cocaine (vehicle or 15 mg/kg i.p.). In a separate group of animals the effects of systemic and intra-NAc adenosine A_{2A} receptor blockade on cocaine-primed reinstatement was tested by a pretreatment of the adenosine A_{2A} antagonist, MSX-3 (5 and 10 mg/side), 5 min prior to a priming injection of cocaine (vehicle, 5, or 10 mg/kg i.p.).

Effects of A_{2A} receptor stimulation or blockade on D_2 agonist-primed reinstatement

The effect of intra-NAc adenosine A_{2A} receptor stimulation on dopamine D_2 receptor-primed relapse behavior was assessed by a pretreatment of the adenosine A_{2A} agonist, CGS 21680 (vehicle or 2.5 ng/side), administered 5 min prior to quinpirole

treatment (0.3 mg/kg). The effect of intra-NAc adenosine A_{2A} receptor antagonism on dopamine D_2 receptor-primed relapse behavior was assessed by administration of a pretreatment of the adenosine A_{2A} antagonist, MSX-3 (vehicle and 10 ug/side, intra-NAc), 5 min prior to quinpirole treatment (vehicle, 0.1, 0.3, and 1.0 mg/kg, i.p.).

Sucrose Reinstatement

Animals were trained to self-administer sucrose pellets on an FR1:TO 20 sec schedule as described above. After 15 daily sessions (50 pellets/session), animals remained in their home cages for 7 days of "abstinence", and were then subjected to extinction training in five daily 4-hr sessions. Following extinction training, animals were tested for reinstatement of sucrose seeking. A pretreatment of CGS 21680 (2.5 ng/side, intra-NAc microinfusion) was administered 5 min prior to sucrose reinstatement testing. Reinstatement testing was initiated by non-contingent sucrose pellet delivery in a single 2-hr test immediately following 2-hr of extinction conditions. During the reinstatement phase, animals were presented with the non-contingent delivery of a sucrose pellet every 2 min for the first 10 min of the session (total of 5 pellets). Responding at both levers was recorded, but resulted in no cues or sucrose pellet delivery.

Locomotor Testing

Locomotor activity was recorded in plexiglass chambers (San Diego Instruments) measuring 16x16x15 inches with 16 pairs of photobeams spaced 1 inch apart on both the x and y axes. All locomotor tests were performed in darkened chambers during the

light phase of the light:dark cycle. One week following the completion of the self-administration and reinstatement procedures, animals were habituated to the locomotor testing chambers for 2-hr (1 day prior to cocaine-induced locomotor activity testing). On test day animals were habituated for 1.5-hr, and given a pretreatment of CGS 21680 (vehicle, 2.5 or 5 ng/side, intra-NAc microinfusion). 5 min following the pretreatment, all animals received cocaine (15 mg/kg). Total locomotor activity as measured by number of beam breaks during the 2-hr testing period.

Histology and Microinjections

Microinjections were administered as pretreatments 5 min prior to challenge injections. All microinjections occurred in the NAc at a volume of 0.5-1.0 µL. Infusions occurred over a 1 min period, and the microinjectors were removed 1 min after the full volume of the infusion was given to ensure absorption into the tissues. In these experiments reinstatement was assessed over repeated sessions and animals received a maximum of 5 treatments in a randomized/counter-balanced order. All animals did not receive all treatments due to concerns of residual testing and weakening of reinstatement responding over repeated trials.

After all experimental procedures were complete, rats were euthanized with carbon dioxide gas and 1.0 µL/side of 0.1% cresyl violet was infused intracranially to verify cannulae tip placements. Placements were determined from coronally-sliced sections and recorded on histological maps. Data from rats with incorrect placements were excluded from these studies.

Statistical analyses

The numbers of animals in each group ranged from 4-17 and are reported for each experiment in the figure captions. All reinstatement data (dependent variables: active lever and inactive lever responses) were analyzed by a 2-way ANOVA with lever (within) and treatments with A_{2A} agonists/antagonist-cocaine/quinpirole (between) as the factors unless otherwise noted. Significant interactions were followed up with simple main effects analyses (1-way ANOVA) and post hoc tests (Bonferrroni's comparisons). Sucrose reinstatement data were analyzed by two separate 2-way ANOVAs with session (within) and the CGS-21680/cocaine treatment (between) as the factors. Significant effects were followed up with appropriate post hoc tests. The effect of CGS-21860 pretreatment on cocaine-induced locomotor activity was analyzed by 1-way between subjects ANOVA. Statistical significance was set at $p<0.05$ for all tests.

Results

Intra-NAc adenosine A_{2A} receptor stimulation dose-dependently blocks cocaine-induced reinstatement

Animals were trained to self-administer cocaine for 3 wks (avg intake: X = 74.0 ± 3.5) and lever responding was extinguished in daily sessions (Figure 2.1a and b). Figure 1c illustrates that an intra-NAc pretreatment of the adenosine A_{2A} agonist CGS 21680 dose-dependently reduces cocaine-induced drug seeking. A significant lever X treatment interaction ($F_{6,72}=8.65$; $p<0.0001$) and significant main effects of lever

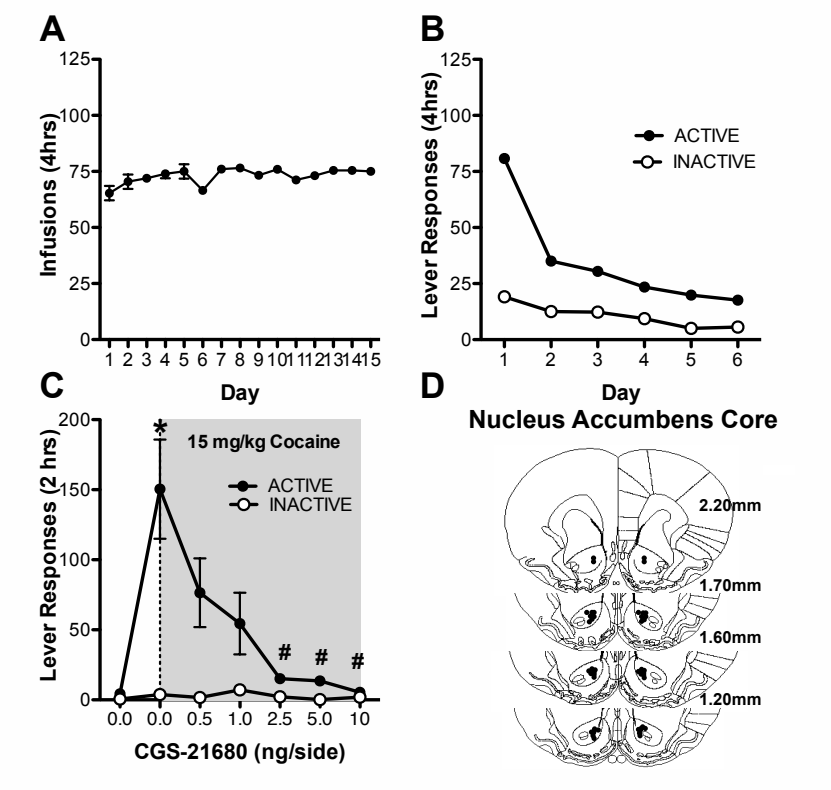

Figure 2.1 Intra-NAc administration of the adenosine A_{2A} agonist CGS 21680 dose-dependently blocked cocaine-induced reinstatement. (a) Average number of cocaine infusions in each 4 h session over the 3 week cocaine self-administration phase. (b) Extinction training was performed in 6 daily 4 h sessions. (c) The adenosine A_{2A} receptor agonist, CGS 21680, dose-dependently reduced cocaine-induced active lever responding. (d) Injection sites of animals included in the data set. Number of animals per treatment group: 0.0 CGS/saline=17, 0.0 CGS/15 mg/kg cocaine=16, 0.5 ng CGS/15 mg/kg cocaine=6, 1.0 ng CGS/15 mg/kg cocaine=7, 2.5 ng CGS/15 mg/kg cocaine=13, 5.0 ng CGS/15 mg/kg cocaine=10, and 10.0 ng CGS/15 mg/kg cocaine=10. *Significant from 0.0 CGS/saline ($p<0.0001$ Bonferroni's post-test); #significant from 0.0 CGS/15 mg/kg cocaine ($p<0.0001$ Bonferroni's post-test).

($F_{1,72}$=27.82; p<0.0001) and treatment ($F_{6,72}$=8.77; p<0.0001) were observed. Subsequent analysis of the interaction found that the cocaine-prime in the absence of CGS 21680 significantly induced active lever pressing, that was dose-dependently decreased by an intra-NAc pretreatment with CGS 21680 ($F_{6,72}$=8.726; p<0.0001). Significant effects of CGS 21680 were also observed on the inactive lever ($F_{6, 72}$=2.929; p<0.05).

Intra-NAc adenosine A_{2A} receptor stimulation blocks D_2 agonist-induced reinstatement

Animals in this experiment averaged 76.2 ± 5.07 cocaine infusions over the last 5 days of self-administration. Figure 2.2a demonstrates that a pretreatment of CGS 21680 (2.5 ng/side) blocks quinpirole-induced reinstatement. A significant treatment x lever interaction ($F_{3, 36}$=23.67; p<0.0001) and significant main effects of treatment ($F_{3, 36}$=24.16; p<0.0001) and lever ($F_{1, 36}$=31.33; p<0.0001) were observed. Simple main effects analysis of the interaction found that quinpirole significantly increased active lever pressing, and that an intra-NAc pretreatment with CGS 21860 prevented this increase ($F_{3, 36}$=24; p<0.0001). A simple main effects analysis of inactive lever and treatment found that quinpirole alone significantly increased inactive lever responding when compared with CGS 21680 alone ($F_{3, 36}$=3.52; p<0.05). While systemic stimulation of adenosine A_{2A} receptors is not sufficient to completely block dopamine D_2 agonist-induced reinstatement, our findings suggest that intra-NAc stimulation of adenosine A_{2A} receptors effectively inhibits quinpirole-induced reinstatement suggesting a localized interaction of adenosine A_{2A} and dopamine D_2 receptors within the NAc.

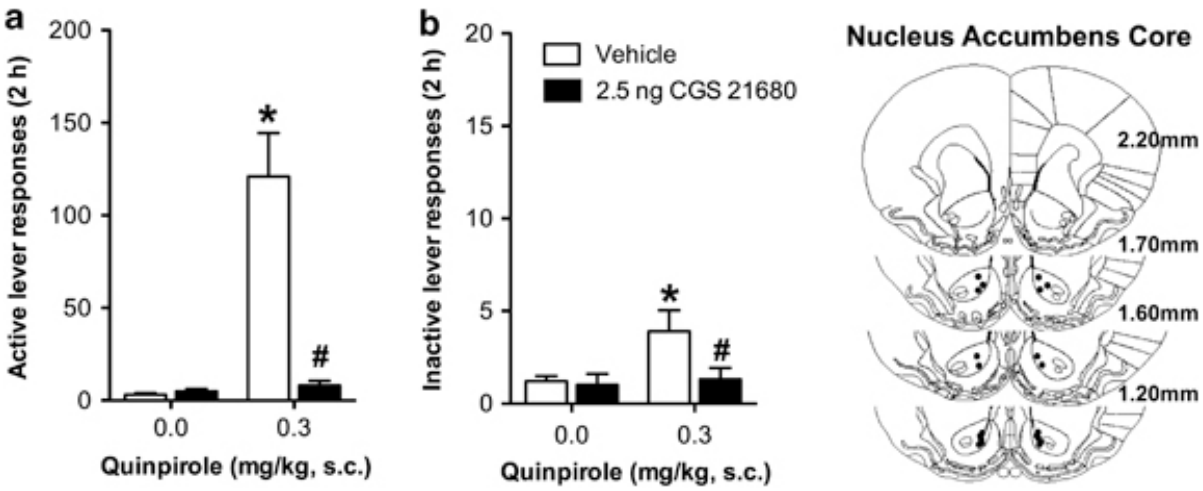

Figure 2.2 Intra-NAc administration of the adenosine A_{2A} agonist CGS 21680 blocks quinpirole-induced reinstatement. (a) An intra-NAc pretreatment of the A_{2A} receptor agonist, CGS 21680 (2.5 ng per side), was sufficient to block D_2 agonist-induced reinstatement. (b) Inactive lever presses from the reinstatement session reveal that quinpirole alone significantly increases inactive lever presses, which was prevented by 2.5 ng CGS 21680. (c) Injection sites for animals included in the data set. Number of animals per treatment group: 0.0 CGS/saline=10, 2.5 ng CGS/saline=10, 0.0 CGS/0.3 mg/kg quinpirole=10, and 2.5 ng CGS/0.3 mg/kg quinpirole=10. *Significant from saline/saline ($p<0.05$ Bonferroni's post-test). #Significant from saline/0.3 mg/kg quinpirole ($p<0.05$ Bonferroni's post-test).

Effects of intra-NAc adenosine A_{2A} receptor stimulation on cocaine-induced locomotor activity

At high doses, systemic adenosine A_{2A} receptor stimulation via CGS 21680 can reduce locomotor activity (Barraco *et al*, 1993, 1994). To ensure that reduced lever responding was not a result of locomotor suppression we assessed the effects two effective doses of intra-NAc CGS 21680 (2.5 ng/side and 5 ng/side) on cocaine-induced locomotor activity. These tests were performed in a subset of animals that self-administered cocaine. Figure 2.3 illustrates that intra-NAc pretreatment of CGS 21680 at either dose does not produce statistically significant reductions in cumulative cocaine-induced locomotor activity over the two hour session ($F_{2, 10}$=1.086, p=0.37). However, qualitative differences in the time-course of cocaine-induced activity were observed at the higher dose (5 ng/side) of CGS 21680 (Figure 2.3b). Analysis of the locomotor time course revealed significant main effects of time ($F_{15, 160}$ = 8.901, p< 0.0001) and group ($F_{2, 160}$ = 5.908, p< 0.01), but no significant time x treatment interaction ($F_{30, 160}$ = 0.3424, p = 0.995). Simple main effects analysis of treatment revealed a significant reduction in locomotor activity of the group receiving 5.0 ng/side CGS 21680 compared with the vehicle group (p < 0.05).

Effects of intra-NAc adenosine A_{2A} receptor stimulation on sucrose-reinstatement

As an additional control for potential motivational effects of adenosine A_{2A} receptor stimulation we examined the effects of the minimally effective dose of CGS 21680 (2.5 ng/side) on reinstatement to sucrose-seeking using non-contingent delivery

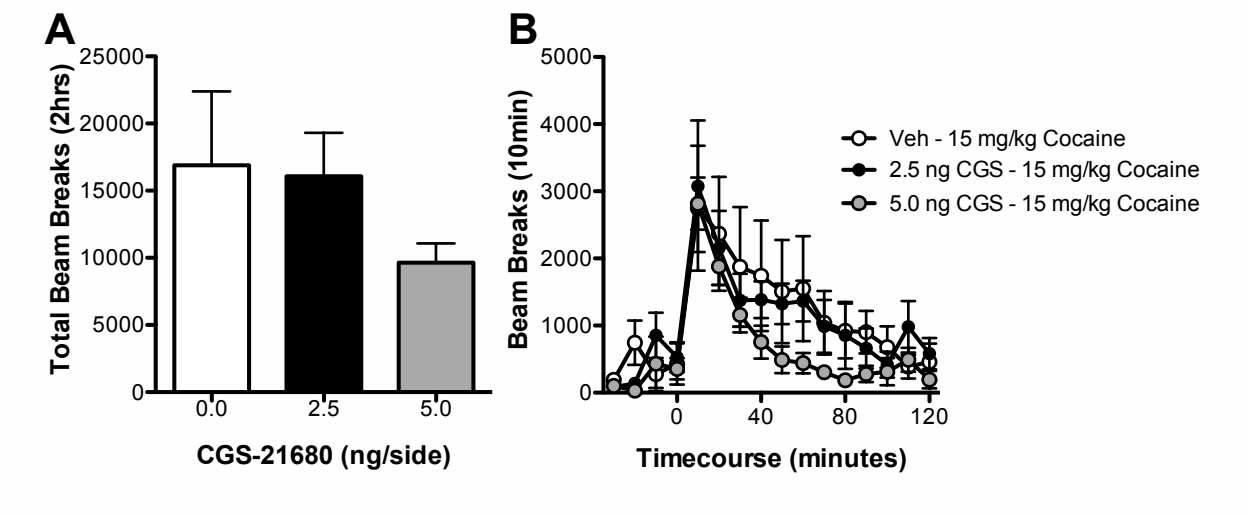

Figure 2.3. Adenosine A_{2A} agonist CGS 21680 does not alter cocaine-induced locomotor behavior. (a) No significant differences were observed in total cocaine-induced locomotor activity over the 2-h test period of animals between receiving a pretreatment with 0.0, 2.5 and 5.0 ng per side CGS 21680 before cocaine (15 mg/kg, intraperitoneally). (b) Time course of locomotor activity illustrating the last 30 min of the habituation period (–30 to 0 min), followed by the effects of 15 mg/kg cocaine (intraperitoneally) with and without a pretreatment of intra-NAc CGS 21680. Note the significant differences in the time course of the cocaine-induced locomotor activity at 5 ng per side CGS 21680 ($p<0.01$ Bonferroni's post-test). Number of animals per treatment group: 0.0 CGS/15 mg/kg cocaine=4, 2.5 ng CGS/15 mg/kg cocaine=5, and 5.0 ng CGS/15 mg/kg cocaine=4.

of sucrose pellets in animals previously trained to self-administer sucrose pellets (Figure 2.4a). Figure 2.4c shows significant sucrose seeking on the active lever in both groups ($F_{1, 12}$=48.71, p<0.0001) that was unaltered by the minimally effective dose of CGS 21860 ($F_{1, 12}$=1.618, p=0.23). A significant increase in inactive lever responding was observed during the reinstatement session compared with the extinction session, however in both the extinction session (p<0.05) and reinstatement session (p<0.0001) active lever pressing was significantly higher than inactive (data not shown). While an intra-NAc infusion of the adenosine A_{2A} receptor agonist, CGS 21680 (2.5 ng/side) is sufficient to block both cocaine- and quinpirole-induced reinstatement, it does not affect reinstatement to natural rewards. This suggests that the effects of the agonist on cocaine- and quipirole-induced reinstatement in cocaine-exposed animals can be disassociated from its effects on sucrose seeking in cocaine-naïve animals.

Systemic and intra-NAc blockade of adenosine A_{2A} receptors moderately reinstate cocaine seeking

Animals in these experiments had an average of 71.44 ± 9.17 cocaine infusions over the last 5 days of self-administration. Figure 2.5a illustrates that a systemic blockade of adenosine A_{2A} receptors with MSX-3 significantly increases active lever pressing in a dose dependent manner. A significant treatment X lever interaction ($F_{2, 31}$=6.545; p<0.01) and significant main effects of treatment ($F_{2, 31}$=5.512; p<0.01) and lever ($F_{1, 31}$=12.8; p<0.01) were observed. Subsequent analysis of the interaction found

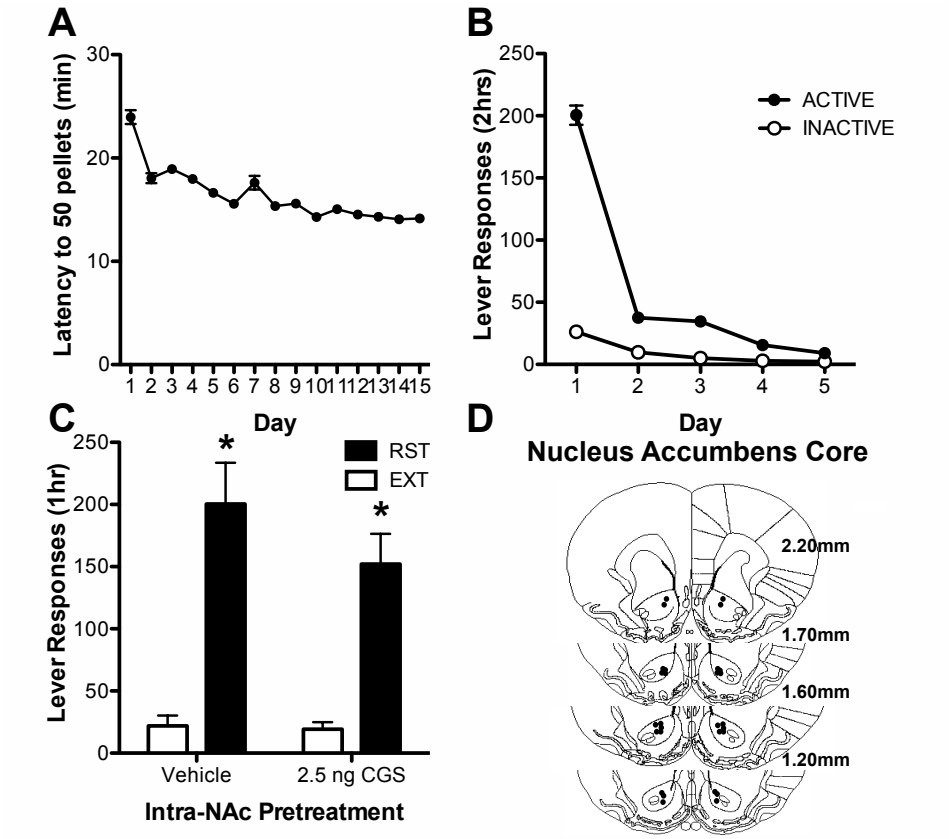

Figure 2.4 Sucrose reinstatement was unaffected by adenosine A_{2A} receptor agonist CGS 21680. (a) Sucrose self-administration was conducted over 3 weeks, and animals' latency to acquire 50 pellets was recorded. (b) Extinction training was performed in 5 daily sessions until active lever responding was reduced to levels comparable with inactive lever responding. (c) Significant sucrose reinstatement was observed compared with extinguished responding; however, an intra-NAc pretreatment of 2.5 ng per side CGS 21860 failed to alter sucrose seeking compared with vehicle control. Active lever responding is shown during the last hour of extinction (white bars, extinction) and the reinstatement phase (black bars, reinstatement). (d) Injection sites of animals included in the data set. Number of animals per treatment group: saline=7 and 2.5 ng CGS=7. *Significant from extinction ($p<0.0001$).

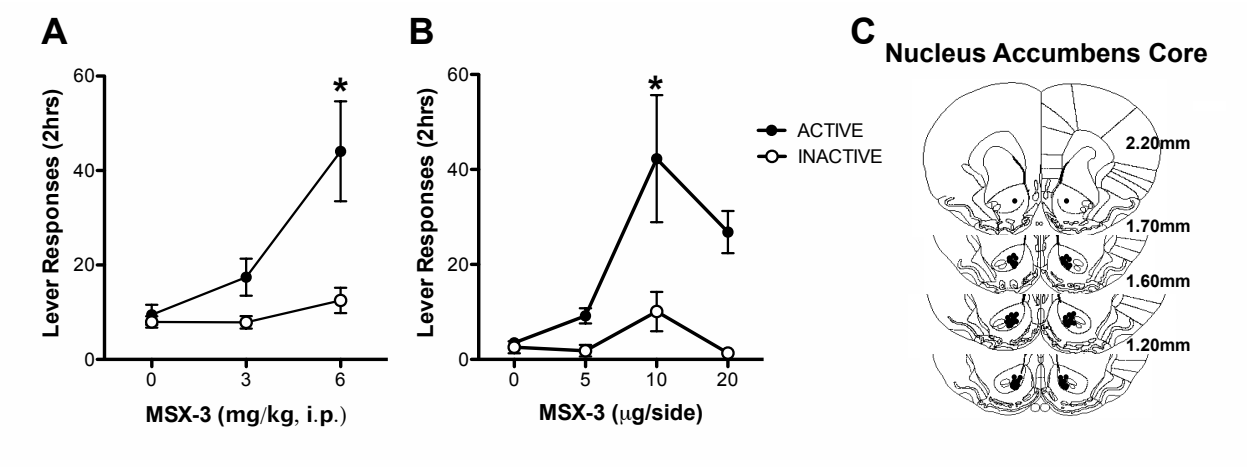

Figure 2.5 Systemic and intra-NAc blockade of adenosine A_{2A} receptors via MSX-3 produces cocaine seeking. (a) Systemic administration of MSX-3 (3 and 6 mg/kg) increased active lever responding in a dose-dependent manner. Number of animals per treatment group: saline=13, 3 mg/kg MSX-3=7, and 6 mg/kg MSX-3=14. (b) Intra-NAc administration of MSX-3 (5, 10, and 20 µg per side) increased active lever responding in a dose-dependent manner. The number of animals per treatment group: saline=14, 5 µg MSX-3=6, 10 µg MSX-3=11, and 20 µg MSX-3=6. (c) Injection sites of animals included in the intra-NAc MSX-3 data set. *Significant from saline ($p<0.01$ Bonferroni's post-test).

that a systemic MSX-3 pretreatment (6 mg/kg) significantly induced active lever pressing ($F_{2, 31}$=6.16; p<0.01). There was no significant effect of systemic administration of MSX-3 on the inactive lever ($F_{2, 31}$=1.666, p=0.21).

Although a systemic blockade of adenosine A_{2A} receptors resulted in a significant increase in active lever responding, the overall reinstatement produced appeared moderate compared to cocaine- and quinpirole-induced cocaine seeking. To determine if a blockade of adenosine A_{2A} receptors localized to the NAc would produce a more robust reinstatement, we assessed the effects of intra-NAc MSX-3 on reinstatement. Animals in these experiments had an average of 69.45 ± 4.5 cocaine infusions over the last 5 days of self-administration. MSX-3 significantly increased active lever pressing in a dose dependent manner, but overall resulted in only a modest reinstatement (Figure 2.5b). A significant treatment X lever interaction ($F_{3, 33}$=4.488; p<0.01) and significant main effects of treatment ($F_{3, 33}$=5.636; p<0.01) and lever ($F_{1, 33}$=16.8; p<0.001) were observed. Subsequent analysis of the interaction found that local microinjections of MSX-3 (10 µg/side) significantly increased active lever pressing ($F_{3, 33}$=5.499; p<0.01). There was no significant effect of the intra-NAc MSX-3 treatment on the inactive lever ($F_{3, 33}$=2.462, p=0.08).

Intra-NAc blockade of adenosine A_{2A} receptors potentiates cocaine- and D_2 agonist-induced reinstatement

Because a blockade of adenosine A_{2A} receptors via MSX-3 alone resulted in only modest reinstatement to cocaine seeking, we hypothesized that an intra-NAc

pretreatment of MSX-3 may potentiate reinstatement to sub-threshold doses of cocaine and quinpirole by enabling more potent stimulation of NAc DA receptor stimulation. Animals in these experiments had an average of 70.02 ± 6.82 cocaine infusions over the last 5 days of self-administration. Figure 2.6b demonstrates that an intra-NAc pretreatment of MSX-3 significantly increased active lever responding to a sub-threshold dose of cocaine (5 mg/kg), which alone does not produce reinstatement. A significant treatment X lever interaction ($F_{4, 58}$=13.07; $p<0.0001$) and main effects of treatment ($F_{4, 58}$=9.279; $p<0.0001$) and lever ($F_{1, 58}$=83.06; $p<0.0001$) were observed. A simple main effects analysis of the interaction found that the pretreatment of MSX-3 significantly increased active lever responding to a sub-threshold dose of cocaine ($F_{4, 58}$=10.98; $p<0.0001$). While significant effects of treatment on the inactive lever were observed ($F_{4, 58}$=2.735, $p<0.05$), post hoc testing revealed no significant differences between treatment groups.

Additionally, we examined the effect of adenosine A_{2A} receptor blockade on reinstatement induced by the dopamine D_2 agonist, quinpirole, to determine if removing the tonic inhibition of the adenosine A_{2A} receptor over the dopamine D_2 receptor could enhance responding to dopamine D_2 receptor stimulation. Animals in these experiments had an average of 69.92 ± 4.09 cocaine infusions over the last 5 days of self-administration. Figure 2.6c illustrates that an intra-NAc pretreatment of MSX-3 potentiates active lever responding to a sub-threshold dose of quinpirole (0.1 mg/kg), which alone does not significantly increase active lever responding when compared with the vehicle-saline control. A significant treatment X lever interaction ($F_{7, 97}$=5.86;

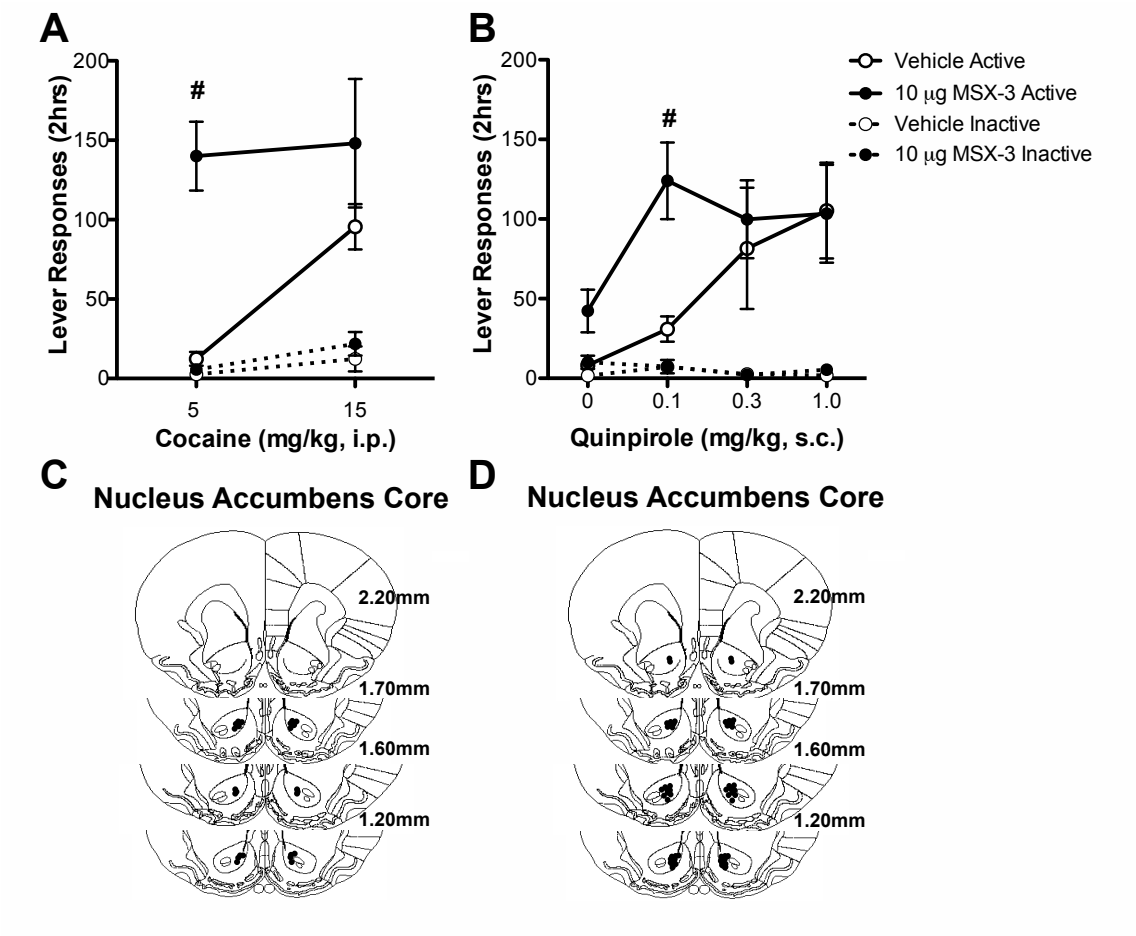

Figure 2.6 Intra-NAc blockade of adenosine A_{2A} receptors via MSX-3 potentiates reinstatement response to sub-threshold doses of cocaine and quinpirole. (a) An intra-NAc pretreatment with 10 µg per side MSX-3 potentiated active lever responding at a sub-threshold dose of cocaine (5 mg/kg) compared with vehicle pretreatment. #Significant from saline/5 mg/kg cocaine ($p<0.0001$ Bonferroni's post-test). The number of animals per treatment group: vehicle/saline=13, vehicle/5 mg/kg cocaine=13, vehicle/15 mg/kg cocaine=13, 10 µg MSX-3/5 mg/kg cocaine=12, and 10 µg MSX-3/15 mg/kg cocaine=12. (b) Injection sites of animals shown in MSX-3 effects on cocaine-induced reinstatement. (c) An intra-NAc pretreatment of MSX-3 (10 µg per side) significantly increases active lever responding at a sub-threshold dose of quinpirole (0.1 mg/kg) compared with vehicle pretreatment. #Significant from saline/0.1 mg/kg quinpirole ($p<0.05$ Bonferroni's post-test). The number of animals per treatment group: vehicle/saline=29, vehicle/0.1 mg/kg quinpirole=12, vehicle/0.3 mg/kg quinpirole=5, vehicle/1.0 mg/kg quinpirole=13, 10 µg MSX-3/saline=11, 10 µg MSX-3/0.1 mg/kg quinpirole=11, 10 µg MSX-3/0.3 mg/kg quinpirole=12, and 10 µg MSX-3/1.0 mg/kg quinpirole=12. (d) Injection sites of animal included in MSX-3 effects on quinpirole-induced reinstatement.

$p<0.0001$) and main effects of treatment ($F_{7, 97}=5.863$; $p<0.0001$) and lever ($F_{1, 97}=88.87$ $p<0.0001$) were observed. A subsequent analysis of the interaction revealed that an intra-NAc pretreatment of MSX-3 significantly increased active lever responding to a sub-threshold dose of quinpirole ($F_{7, 97}=5.908$; $p<0.0001$). Again, significant effects of treatment on the inactive lever were observed ($F_{7, 97}=2.138$, $p<0.05$), however subsequent post hoc testing revealed no significant differences between treatment groups.

Discussion

We have previously shown that systemic adenosine A_{2A} receptor stimulation attenuates cocaine seeking induced by pharmacological stimuli and drug related cues (Bachtell *et al*, 2009). Here we elucidate the NAc as a primary site of action for these effects. Our findings reveal that pharmacological manipulation of adenosine A_{2A} receptors within the NAc bi-directionally alters cocaine seeking in extinguished rats. We show that intra-NAc stimulation of adenosine A_{2A} receptors attenuates cocaine seeking induced by pharmacological stimuli such as cocaine and quinpirole suggesting that adenosine A_{2A} receptors represent a potential target for therapies aiming to curb relapse vulnerability. Because systemic and higher doses of intra-NAc adenosine A_{2A} agonists reduce lever pressing for sucrose (Font *et al*, 2008) and reduce locomotor activity (Barraco *et al*, 1993, 1994), we examined the effects of the minimally effective CGS 21680 dose on sucrose seeking. We show that it is specific to cocaine, since adenosine A_{2A} stimulation did not significantly reduce sucrose seeking. Further support

for this comes from a recent study showing that an accumbens specific knockout of adenosine A_{2A} receptors does not alter wakefulness at baseline conditions (Lazarus *et al*, 2011).

We also demonstrate that intra-NAc blockade of adenosine A_{2A} receptors produces modest cocaine seeking alone. However, combining intra-NAc blockade of adenosine A_{2A} receptors with sub-threshold doses of cocaine and quinpirole results in robust cocaine seeking, suggesting that removing the inhibitory control that the adenosine A_{2A} receptor exerts over the dopamine D_2 receptor allows a normally ineffectual dose of cocaine or quinpirole to induce reinstatement. Other models support this tonic inhibitory role of adenosine A_{2A} receptors in behavioral regulation. For example, a recent study demonstrated that blocking adenosine A_{2A} receptors and hence, removing the adenosine "brake", produces wakefulness (Lazarus *et al*, 2011). Antagonism of adenosine A_{2A} receptors also restores deficits in effort-related behaviors induced by dopamine D_2 receptor blockade (Nunes *et al*, 2010; Worden *et al*, 2009), suggesting that adenosine A_{2A} receptors are a tonic modulator of dopamine D_2 receptor expressing neurons within the striatum (Harper *et al*, 2006; Nagel *et al*, 2003). Our data provide further support that adenosine A_{2A} receptors exert tonic regulation of dopamine D_2 receptors and suggests that adenosine A_{2A} receptors are an important modulator of DA-mediated behavior (Farrar *et al*, 2010; Hakansson *et al*, 2006; Harper *et al*, 2006; Nagel *et al*, 2003; Weber *et al*, 2010).

These findings agree with previous work showing that stimulation of adenosine A_{2A} receptors counteracts cocaine-mediated behaviors, while antagonism augments

cocaine-mediated behaviors. Administration of an adenosine A_{2A} agonist attenuates both the development and expression of behavioral sensitization to cocaine (Filip *et al*, 2006), impairs the acquisition of cocaine self-administration (Knapp *et al*, 2001), and reduces the expression of cocaine conditioned place preference (Poleszak and Malec, 2002a). Blockade of adenosine A_{2A} receptors, on the other hand, enhances both acute and sensitized cocaine-induced locomotor activity (Filip *et al*, 2006), and enhances discriminative stimulus effects of both cocaine and methamphetamine (Justinova *et al*, 2003). Antagonism of adenosine A_{2A} receptors during withdrawal also has reward related effects. Blocking adenosine A_{2A} receptors during a brain stimulation reward task reversed the elevated reward threshold produced by cocaine withdrawal, suggesting that removing the tonic activity of adenosine A_{2A} receptors enables DA signaling to restore reward deficits observed during drug withdrawal (Baldo *et al*, 1999). This explanation is supported by our findings that adenosine A_{2A} receptor blockade produces cocaine seeking by enabling DA receptor stimulation at sub-threshold doses of both cocaine and a dopamine D_2 agonist. Together, these findings indicate that pharmacological stimulation of adenosine A_{2A} receptors opposes the behavioral effects of cocaine while pharmacological blockade of adenosine A_{2A} receptors enhances cocaine's effects.

Studies utilizing genetic deletion of adenosine A_{2A} receptors generally show effects opposite to those reported with pharmacological blockade of adenosine A_{2A} receptors. In fact, adenosine A_{2A} receptor knockout mice display reduced locomotor activity to acute injections of amphetamine and cocaine and impaired development of

amphetamine sensitization (Chen *et al*, 2003). In addition, adenosine A_{2A} receptor knockout mice show reduced responding for cocaine on an FR1, FR3 and progressive ratio schedule of reinforcement (Chen *et al*, 2000; Chen *et al*, 2003; Soria *et al*, 2006). It is possible that compensatory changes during development or the lack of neuroanatomical specificity of the adenosine A_{2A} receptor knockout contribute to these conflicting results between the two experimental methods. Indeed, a recent study showed striatal-specific knockdown of adenosine A_{2A} receptors enhances locomotor activity in response to cocaine, while a forebrain-specific knockdown of the adenosine A_{2A} receptors reduces cocaine-induced locomotor activity (Shen *et al*, 2008). Our experiments corroborate these findings by demonstrating that adenosine A_{2A} receptor blockade specifically in the NAc enhances cocaine seeking. Taken together, these findings suggest that adenosine A_{2A} receptors localized in the striatum and NAc provide inhibitory control over cocaine-mediated behaviors such as cocaine seeking as suggested by the pharmacological manipulations of adenosine A_{2A} receptors.

It should be emphasized that the present experiments targeted the medial division of the NAc core, an area is known to be involved in the reinstatement of cocaine seeking (Bachtell *et al*, 2005; Ito *et al*, 2004; McFarland *et al*, 2003). Recently, the NAc has been discussed in terms of a medial-lateral continuum based on “spiraling” dopaminergic innervation and functional consequences (Haber *et al*, 2000; Heimer *et al*, 1997; Ikemoto *et al*, 2005). Modulation of the dopamine input along this medial-lateral continuum supports these functional differences in cocaine seeking. Thus, manipulations of dopamine receptors in the medial divisions of the NAc (shell and

medial core) induce cocaine seeking, while similar manipulations in the lateral NAc core do not regulate cocaine seeking (Bachtell *et al*, 2005; Schmidt *et al*, 2006a; Schmidt *et al*, 2006b). Here, we show that increasing and decreasing adenosine receptor activity in the medial NAc core, is sufficient to inhibit and promote cocaine seeking, respectively.

The NAc is comprised primarily of medium spiny GABAergic neurons that include two distinct subpopulations of neurons that are differentiated by their cellular peptide expression, receptor subtype expression and unique projection targets (Aubert *et al*, 2000; Steiner *et al*, 1998). Dopamine D_1 receptors are found mainly on dynorphin/substance P-expressing neurons that comprise the direct pathway, while dopamine D_2 receptors occur mainly on enkephalin-expressing neurons that comprise the indirect pathway (Lu *et al*, 1998). DA stimulation of both populations in the NAc elicits cocaine seeking (Bachtell *et al*, 2005; Schmidt *et al*, 2006a; Schmidt *et al*, 2006b). Thus, tempering DA signaling in the NAc is an ideal way to prevent relapse. Adenosine A_{2A} receptors are co-localized with dopamine D_2 receptors where they provide reciprocal regulation of dopamine D_2 receptors making them a suitable target to temper DA signaling (Canals *et al*, 2003; Ferre, 1997; Fuxe *et al*, 2003; Hillion *et al*, 2002; Svenningsson *et al*, 1999a; Svenningsson *et al*, 1998; Svenningsson *et al*, 1999b).

Adenosine A_{2A} and dopamine D_2 receptors interact to alter signaling of medium spiny GABAergic neurons within the striatum through several mechanisms. For example, these receptors form heteromeric receptor complexes through electrostatic interactions (Canals *et al*, 2003; Fuxe *et al*, 2003; Hillion *et al*, 2002). Heteromeric formation of A_{2A}-D_2 receptors allows adenosine A_{2A} receptor stimulation to inhibit ligand

binding to dopamine D_2 receptors and decrease G-protein coupling at the dopamine D_2 receptor (Ferre *et al*, 1991a; Fuxe *et al*, 1998; Hillion *et al*, 2002; Torvinen *et al*, 2005). As previously mentioned cocaine reduces the expression of the A_{2A}-D_2 heteromer (Marcellino *et al*, 2010), which may underlie some of the changes in behavioral responses following chronic cocaine intake. It will be critical for future studies to determine the impact of chronic cocaine intake on heteromeric A_{2A}-D_2 receptor expression and how selective pharmacological targeting of these heteromers may be relevant behaviorally.

In addition to the contribution of the A_{2A}-D_2 receptor heteromers, adenosine A_{2A} and dopamine D_2 receptors are coupled to excitatory and inhibitory G proteins, respectively. For example, stimulation of adenosine A_{2A} receptors counteracts the effects of dopamine D_2 receptor stimulation on immediate early gene expression (Morelli *et al*, 1994; Svenningsson *et al*, 1999a) and opposes dopamine D_2 receptor-mediated signal transduction in the striatum (Yang *et al*, 1995). Their complementary intracellular signaling also has profound effects on cAMP production and neuronal excitability (Schiffmann *et al*, 2007; Svenningsson *et al*, 1999a; Tozzi *et al*, 2007) suggesting that reciprocal regulation of downstream targets of cAMP (e.g. PKA-mediated phosphorylation targets) may play a role in the modulation of cocaine seeking. While adenosine A_{2A} receptors obviously play a significant role in modulating DA neurotransmission within the striatum, the cellular mechanisms of our effects on cocaine seeking remain obscure. While it is likely that both A_{2A}-D_2 heteromeric receptors and adenosine A_{2A} receptor intracellular signaling contribute to the modulation of these

behaviors, future studies should focus on the independent contributions in determining their role in modulating cocaine-mediated behaviors.

Adenosine A_{2A} receptors are expressed on other cell types in the NAc providing other possible explanations for our results. For example, expression of adenosine A_{2A} receptors on presynaptic glutamatergic terminals is involved in modulating striatal glutamate release and synaptic plasticity (Hettinger *et al*, 2001; Rodrigues *et al*, 2005; Troisi *et al*, 2005). Thus, stimulation of presynaptic adenosine A_{2A} receptors increases striatal glutamate release and blockade of adenosine A_{2A} receptors produces the opposite effect (Corsi *et al*, 2000; Corsi *et al*, 1999). It seems unlikely that our findings would result from adenosine A_{2A}-induced increases in glutamate release since stimulation of AMPA receptors in the NAc induces cocaine seeking, and blockade of AMPA receptors prevents both cocaine- and cue-induced drug seeking (Cornish *et al*, 1999). There is also evidence that adenosine A_{2A} receptors are expressed on cholinergic interneurons (Tozzi *et al*, 2011), although this report conflicts with a previous study where adenosine A_{2A} receptor mRNA was absent in cholinergic interneurons (Svenningsson *et al*, 1997). Cholinergic interneurons make up a small percentage (<5%) of the cell types in the NAc, but have significant effects on modulating both direct and indirect output pathways from the NAc (Kawaguichi *et al*, 1995; Tepper and Bolam, 2004). It was recently shown that simultaneous blockade of adenosine A_{2A} receptors and stimulation of dopamine D_2 receptors decreases firing of cholinergic interneurons, which consequently reduces the muscarinic M_1 receptor activity on medium spiny GABAergic neurons of the striatum (Tozzi *et al*, 2011). Thus, our findings that an

adenosine A_{2A} antagonist enhances cocaine seeking may result from reduced muscarinic activity. Recent work does not support this notion since blockade of muscarinic receptors in the NAc attenuates cocaine seeking (Mark *et al*, 2006; Yee *et al*, 2011). Thus, it is unclear whether our manipulations on adenosine A_{2A} receptors within the NAc are having a large effect on cholinergic interneurons. Future studies will help to elucidate the interactions between adenosine A_{2A} and dopamine D_2 receptors on cholinergic interneurons.

Overall, the results of these experiments suggest an important role of adenosine A_{2A} receptors in the modulation of cocaine seeking in an animal model of relapse. We demonstrate that intra-NAc stimulation of adenosine A_{2A} receptors blocks both cocaine- and quinpirole-induced drug seeking while intra-NAc adenosine A_{2A} receptor blockade enhances cocaine seeking. While, the antagonistic interaction between adenosine A_{2A} and dopamine D_2 receptors on striatal neuronal transmission is supported by these experiments, the relative contribution of heteromeric and non-heteromeric complexes is unknown. Together, our results suggest that interactions between adenosine A_{2A} and dopamine D_2 receptors influence striatal signaling that mediates cocaine seeking, but future studies should examine the specific cellular mechanisms by which adenosine A_{2A} stimulation reduces dopamine D_2 receptor mediated behaviors. Finally, the results of this study illuminate the potential for A_{2A} receptor stimulation as an effective strategy for reducing the relapse susceptibility.

Chapter 3: Opposing effects of pre- and postsynaptic adenosine A_{2A} receptor blockade on cocaine seeking

Abstract

Drug-associated cues or pharmacological stimuli are known to mediate cocaine seeking through enhanced dopamine and glutamate neurotransmission in the NAc. Previous work has shown that adenosine receptors modulate reinstatement to cocaine seeking as well as other cocaine-mediated behaviors. Adenosine A_{2A} receptors are expressed on both pre- and postsynaptic terminals in the NAc. Postsynaptic A_{2A} receptors in the NAc are colocalized on dopamine D_2 expressing medium spiny GABAergic neurons where they reduce dopamine neurotransmission. Presynaptic A_{2A} receptors are expressed on glutamate terminals in the NAc where they enhance glutamate transmission onto MSNs. The goal of these studies was to examine the differential effects of pre- and postsynaptic A_{2A} receptor blockade on cocaine seeking. Rats were trained to lever press for cocaine in daily self-administration sessions on a fixed-ratio 1 schedule for 10 consecutive days. After one day of abstinence, lever pressing was extinguished in 8-10 daily extinction sessions. We subsequently identified whether a systemic administration of a presynaptic A_{2A} receptor antagonist, SCH 442416, and a postsynaptic A_{2A} receptor antagonist, KW 6002, would reinstate cocaine seeking. Higher doses of KW 6002 (0.3, 1.0, 3.0 mg/kg, i.p.) induced cocaine seeking, while SCH 442416 (1.0, 3.0 mg/kg, i.p.) had no effect. We next examined the effect of pre- and postsynaptic A_{2A} receptor blockade on cocaine-induced reinstatement. Systemic

administration of cocaine (15 mg/kg, i.p.) produced robust reinstatement that was blunted by pretreatment with the presynaptic A_{2A} antagonist, SCH 442416 (1.0 mg/kg, i.p.). On the other hand, blockade of postsynaptic A_{2A} receptors by KW 6002 (0.3 mg/kg, i.p.) amplified cocaine-induced reinstatement (5 and 15 mg/kg, i.p.). We hypothesize that SCH 442416 reduces reinstatement by dampening excessive cocaine-induced glutamate release in the NAc from the prefrontal cortex (PFC) that is necessary for cocaine seeking. In order to assess this hypothesis, cocaine seeking was induced by either an intra-PFC injection of AMPA (0.8 nM/side) or cocaine (200 mg/side). A systemic pretreatment with the presynaptic A_{2A} antagonist, SCH 442416 (1.0 mg/kg, i.p.) significantly reduced both intra-PFC AMPA and cocaine induced reinstatement. These findings suggest that presynaptic A_{2A} receptors may be a viable target in tempering the augmented glutamate release that plays a key role in driving reinstatement.

Introduction

A major obstacle in the successful treatment of cocaine addiction is the persistence of cocaine craving and prevalence of relapse. In rodents, chronic cocaine self-administration has been shown to alter glutamate and dopamine signaling, and these neurotransmitter systems play a significant role in cocaine seeking (Cornish *et al*, 2001; Kalivas, 2004; Schmidt *et al*, 2005; Schmidt *et al*, 2010). Increased glutamate transmission in the NAc is both necessary and sufficient for reinstatement of cocaine seeking (Cornish *et al*, 2001; McFarland *et al*, 2003). Similarly, dopamine D_1 and D_2 receptors in the NAc also play a significant role in reinstatement of cocaine seeking (Anderson *et al*, 2003; Anderson *et al*, 2006; Bachtell *et al*, 2005; Schmidt *et al*, 2006a). Developing novel pharmacotherapies that modulate the signaling of these systems may be key to decreasing relapse susceptibility in addicts.

There is clear evidence that adenosine acts as a modulator of both glutamate and dopamine through actions at two receptor subtypes expressed in the brain, adenosine A_{2A} and A_1 receptors (Goodman and Synder, 1982; Svenningsson *et al*, 1997). Adenosine A_{2A} receptors are most densely expressed in the striatum (Dixon *et al*, 1996). Within the accumbens, postsynaptic adenosine A_{2A} receptors are co-localized on dopamine D_2 expressing indirect pathway medium spiny GABA neurons (MSNs) (Fink *et al*, 1992; Svenningsson *et al*, 1999b). Postsynaptic adenosine A_{2A} receptor stimulation opposes dopamine D_2 receptor signaling (Ferre *et al*, 1991b), and several studies have shown that adenosine A_{2A} receptors exert tonic inhibition over dopamine D_2 receptors (Farrar *et al*, 2010; Hakansson *et al*, 2006; Harper *et al*, 2006).

Previous work suggests that adenosine A_{2A} receptors can regulate cocaine behaviors. For example, stimulation of adenosine A_{2A} receptors reduces sensitization to cocaine (Filip *et al*, 2006; Hobson *et al*, 2012), acquisition of cocaine self-administration (Knapp *et al*, 2001), and reinstatement to cocaine seeking (Bachtell *et al*, 2009; O'Neill *et al*, 2012). In contrast, blockade of adenosine A_{2A} receptors enhances cocaine sensitization (Filip *et al*, 2006) and augments reinstatement responding induced by cocaine or a dopamine D_2 receptor agonist (O'Neill *et al*, 2012). These effects are thought to arise from the antagonistic interactions between postsynaptic adenosine A_{2A} receptors and dopamine D_2 receptors.

Phasic increases in glutamate are mediated by presynaptic adenosine A_1 and A_{2A} receptors in the nucleus accumbens (Orru *et al*, 2011b). Stimulation of adenosine A_1 receptors decreases glutamate release, while stimulation of adenosine A_{2A} receptors enhances the release of glutamate (Ciruela *et al*, 2006; Orru *et al*, 2011b; Quiroz *et al*, 2009). Interestingly, presynaptic adenosine A_{2A} receptors are preferentially located on presynaptic glutamate terminals that synapse onto dopamine D_1 receptor expressing MSNs (Quiroz *et al*, 2009) and there has been recent interest in how presynaptic adenosine A_{2A} receptors contribute to striatal signaling (Ciruela *et al*, 2006; Orru *et al*, 2011a). In the dorsal striatum, administration of an antagonist that targets adenosine A_{2A} receptors expressed presynaptically decreased glutamate release evoked by electrical stimulation of the cortex (Orru *et al*, 2011a). Additionally, binding assays for several other adenosine A_{2A} receptor antagonists were performed, and SCH 442416 exhibited a much lower affinity for binding in adenosine A_{2A} receptor and dopamine D_2

receptor expressing cells compared to cells expressing only adenosine A_{2A} receptors (Orru *et al*, 2011a). Interestingly, a recent paper has found that a presynaptic adenosine A_{2A} receptor antagonism shifts THC self-administration dose response curves to the left, consistent with a decrease in its reinforcing effects, while postsynaptic adenosine A_{2A} receptor antagonism resulted in a shift to the left (Justinova *et al*, 2014). Previous work from our lab has also shown that blockade of presynaptic adenosine A_{2A} receptors during extinction training results in lasting decreases in susceptibility to drug-primed reinstatement (O'Neill *et al*, 2014).

The studies presented here distinguish the effects of antagonizing presynaptic vs. postsynaptic adenosine A_{2A} receptors on cocaine seeking. Given the ability of presynaptic adenosine A_{2A} receptor blockade to reduce glutamate release (Orru *et al*, 2011a), and postsynaptic adenosine A_{2A} receptor blockade to facilitate dopamine D_2 receptor signaling (Farrar *et al*, 2010; Ferre *et al*, 1994a; Fuxe *et al*, 2007a), we predict that these antagonists will produce bidirectional effects on cocaine seeking. Thus, we hypothesize that presynaptic adenosine A_{2A} receptor antagonism will reduce reinstatement of cocaine seeking and postsynaptic A_{2A} receptor blockade will enhance cocaine seeking.

Methods

Animals

Male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing between 300-350 grams were individually housed with food and water available *ad libitum*.

Experiments were conducted during the light period of a 12-h light/dark cycle in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the University of Colorado Boulder.

Surgery

Surgical implantation of jugular catheters and intracranial guide cannulae were implanted under halothane anesthesia (1-2.5%). Jugular catheter implantation was performed according to previously published methodology (O'Neill *et al*, 2012). Following jugular catheter implantation, some rats (see experimental procedures below) were fitted into a stereotaxic instrument where the scalp was incised and retracted. The head was positioned to place bregma and lambda at the same depth coordinate. Titanium screws were secured into the skull and holes were drilled allowing bilateral insertion of guide cannula into either the NAc shell (A/P: +1.7; M/L: ±0.8; D/V: -5.7 from bregma) or the mPFC (A/P: +2.7; M/L: ±0.6; D/V: -3.2 from bregma) (Paxinos *et al*, 1998). The guide cannula was then fixed in place by dental cement and dummy stylets extending 1 mm beyond the tip of the guide cannulae were inserted to maintain patency. Following surgery animals were administered (S)-(+)-ketoprofen (5 mg/kg), a non-steroidal anti-inflammatory analgesic (Carabaza *et al*, 1996) and Baytril (enrofloxacin) (5 mg/kg), a fluoroquinolone antibiotic (Vancutsem *et al*, 1990). Catheters were flushed daily with 0.1 mL of heparinized saline and rats recovered for a minimum of 4 days before experimental procedures began.

Drugs

The postsynaptic adenosine A_{2A} receptor antagonist, KW 6002 ((E)-8-(3,4-Dimethoxystyryl)-1,3-diethyl-7-methylxanthine, 8-[(1E)-2-(3,4-Dimethoxyphenyl)ethenyl]-1,3-diethyl-3,7-dihydro-7-methyl-1H-purine-2,6-dione), presynaptic adenosine A_{2A} receptor antagonist, SCH 442416 (2-(2-Furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo [4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine; 5-amino-7-(3-(4-methoxyphenyl)propyl)-2-(2 furyl)pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; 5-Amino-7-[3-(4-methoxy)phenylpropyl]-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine), AMPA receptor agonist, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), and cocaine hydrochloride were obtained from Sigma-Aldrich (St Louis, MO). All drugs were dissolved in sterile-filtered physiological (0.9%) saline, except SCH 442416 and KW 6002. SCH 442416 was dissolved in 33% DMSO and 66% ddH_20. KW 6002 was dissolved in 50% DMSO and 50% ddH_20.

Cocaine Self-Administration, Extinction and Reinstatement Procedures

Self-administration procedures were performed in operant conditioning chambers (Med-Associates, St Albans, VT) equipped with two response levers and an infusion pump system. Animals were initially trained to lever press for sucrose pellets to facilitate acquisition of cocaine self-administration (O'Neill *et al*, 2012). After lever-press training, animals were fed *ad libitum* for at least 24 hr before the catheter and intra-cranial cannula implantation surgery.

Following recovery from surgery, animals were allowed to self-administer intravenous cocaine (0.5 mg/kg/100 µl injection) on a fixed ratio 1 (FR1) reinforcement schedule in daily 2 hr sessions for 10 consecutive days. Cocaine injections were delivered over 5 sec concurrent with the illumination of a cue light above the active lever followed by an additional 15 sec time out period (TO 20 sec) when the house light remained off and responding was without consequence. Inactive lever responses produced no consequence throughout testing.

Twenty-four hours after the last self-administration session, animals underwent 8 daily extinction sessions. Extinction sessions occurred in the absence of cocaine reinforcement in 2 hr test sessions. Thus, responses on the lever previously paired with cocaine injections during self-administration (active lever) and on the inactive lever were recorded, but had no programmed drug or cue delivery. In all experiments, each reinstatement session was initiated with 2 hr of extinction conditions, followed by a 2 hr reinstatement test period. Reinstatement was initiated by a variety of manipulations (see below). During both the extinction phase and reinstatement phase, responses at both the previously active and inactive levers were recorded, but resulted in no cue or drug delivery during testing.

Adenosine A_{2A} Antagonist-Primed Reinstatement

Following a 2 hr extinction session animals were given systemic injections of either the presynaptic adenosine A_{2A} antagonist, SCH 442416 (vehicle, 1.0 mg/kg, and 3.0 mg/kg, i.p.), or the postsynaptic adenosine A_{2A} antagonist, KW 6002 (vehicle, 0.3

mg/kg, 1.0 mg/kg, or 3.0 mg/kg, i.p.). After 5 min, animals were returned to the self-administration chambers for a 2 hr test period. Animals were tested under most doses in a randomized order and received a maximum of 4 treatments. Responses at both levers were recorded, but resulted in no cue or cocaine delivery.

Effects of Pre- and Postsynaptic A_{2A} Receptor Blockade on Cocaine-Primed Reinstatement

The effects of presynaptic A_{2A} receptor blockade on cocaine-primed reinstatement were examined by giving a systemic pretreatment of SCH 442416 (vehicle or 1.0 mg/kg, i.p.) followed by cocaine (vehicle or 15 mg/kg, i.p.) 5 minutes later. After the administration of cocaine, animals were immediately placed in the self-administration chambers for the 2 hr reinstatement test period. The effects of postsynaptic A_{2A} receptor blockade on cocaine-primed reinstatement were tested by giving a systemic pretreatment with KW 6002 (vehicle or 0.3 mg/kg, i.p.) followed, 5 min later, by a systemic injection of cocaine (vehicle, 5 mg/kg, or 15 mg/kg, i.p.). We chose the 5 mg/kg cocaine dose and 0.3 mg/kg KW 6002 dose since neither produce reliably reinstatement and could reveal a synergistic effect on reinstatement responding. Animals were then immediately returned to the operant chambers for a 2 hr reinstatement session.

Effects of Pre- and Postsynaptic A_{2A} Receptor Blockade on Reinstatement Induced by Cortical Stimulation

We next examined the effects of presynaptic and postsynaptic A_{2A} receptor blockade on cortical stimulation-induced reinstatement. Animals were pretreated with systemic injections of either the presynaptic adenosine A_{2A} antagonist, SCH 442416 (vehicle or 1.0 mg/kg, i.p.), or the postsynaptic adenosine A_{2A} antagonist, KW 6002 (vehicle or 0.3 mg/kg, i.p.) prior to intra-PFC infusions of either AMPA (0.8 nM/side) or cocaine (200 µg/side). Following microinjection, animals were placed into the self-administration chambers for the 2 hr reinstatement test.

Effects of Pre- and Postsynaptic A_{2A} Receptor Blockade on Reinstatement Induced by Nucleus Accumbens Stimulation

The effects of presynaptic and postsynaptic A_{2A} receptor blockade on reinstatement induced by stimulation of the nucleus accumbens was also examined. Animals were pretreated with systemic injections of either the presynaptic adenosine A_{2A} antagonist, SCH 442416 (vehicle or 1.0 mg/kg, i.p.), or the postsynaptic adenosine A_{2A} antagonist, KW 6002 (vehicle or 0.3 mg/kg, i.p.) prior to intra-NAc infusion of AMPA (0.8 nM/side) or cocaine (200 µg/side).

Microinjections and Histology

All microinjections were delivered to the PFC or NAcc at a volume of 0.5 µl over a 1-min period. The microinjectors were remained in the cannula for 1 min after the full volume of the infusion was given to ensure absorption of drug into the tissues. After completion of each experiment, rats were euthanized with a Fatal-Plus solution and 1.0

µl of 0.1% cresyl violet was infused bilaterally to verify cannulae tip placements. Placements were determined from coronally sliced sections and recorded on histological maps. Data from rats with incorrect placements were excluded from these studies.

Statistical Analyses

Reinstatement data (dependent variables: active lever and inactive lever responses) were analyzed by a two-way ANOVA with lever (within) and treatments with A_{2A} pre/post antagonists–AMPA/cocaine (between) as the factors, unless otherwise noted. Significant interactions were followed up with post hoc tests (Bonferroni's comparisons). Statistical significance was set at $p<0.05$ for all tests.

Results

Systemic Blockade of Postsynaptic Adenosine A_{2A} Receptors Produces and Enhances Cocaine-Induced Reinstatement

Animals were trained to self-administer cocaine for 10 days (average intake: 42.0 ± 3.9) and lever responding was extinguished in daily sessions (Figure 3.1a and b). Figure 3.1c shows that administration of KW 6002, a postsynaptic adenosine A_{2A} receptor antagonist, dose dependently increased cocaine seeking. A significant treatment X lever interactions ($F_{3,81}=9.37$, $p<0.0001$), and significant main effects of treatment ($F_{3,81}=9.63$, $p<0.0001$) and lever ($F_{1,81}=49.01$, $p<0.0001$) were observed. Post hoc testing revealed that KW 6002 (1.0 and 3.0 mg/kg) significantly increased active

lever pressing compared to vehicle (1.0 mg/kg: t_{81}=4.57, p<0.001; 3.0 mg/kg: t_{81}=4.37, p<0.001) and the lowest dose (0.3 mg/kg) of KW 6002 (1.0 mg/kg: t_{81}=3.45, p<0.01; 3.0 mg/kg: t_{81}=3.66, p<0.001). There was no significant effect of systemic KW 6002 administration on the inactive lever.

We also examined the effect of postsynaptic adenosine A_{2A} receptor blockade on cocaine-induced drug seeking. Animals in this experiment had an averaged 39.6 ± 4.3 cocaine infusions over the last 5 days of cocaine self-administration (data not shown). Pretreatment with KW 6002, at a dose with no effect on cocaine seeking, enhanced reinstatement responding to a cocaine prime (Figure 3.1d). Analysis revealed a significant treatment X lever interaction ($F_{3,31}$=3.94, p<0.05) and significant effects of treatment ($F_{3,31}$=3.91, p<0.05) and lever ($F_{1,31}$=23.39, p<0.0001). Post hoc testing found that 15mg/kg of cocaine increased active lever pressing compared to only 5 mg/kg cocaine (t_{31}=2.42, p<0.05), and that treatment with KW 6002 prior to administration of 15 mg/kg cocaine increased active lever presses compared to 15 mg/kg of cocaine alone (t_{31}=3.08; p<0.01). Active lever pressing following treatment with KW 6002 and 15 mg/kg cocaine was also found to be significantly increased compared to KW 6002 and 5 mg/kg cocaine (t_{31}=3.13, p<0.01) and 5 mg/kg cocaine alone (t_{31}=4.76, p<0.001). There

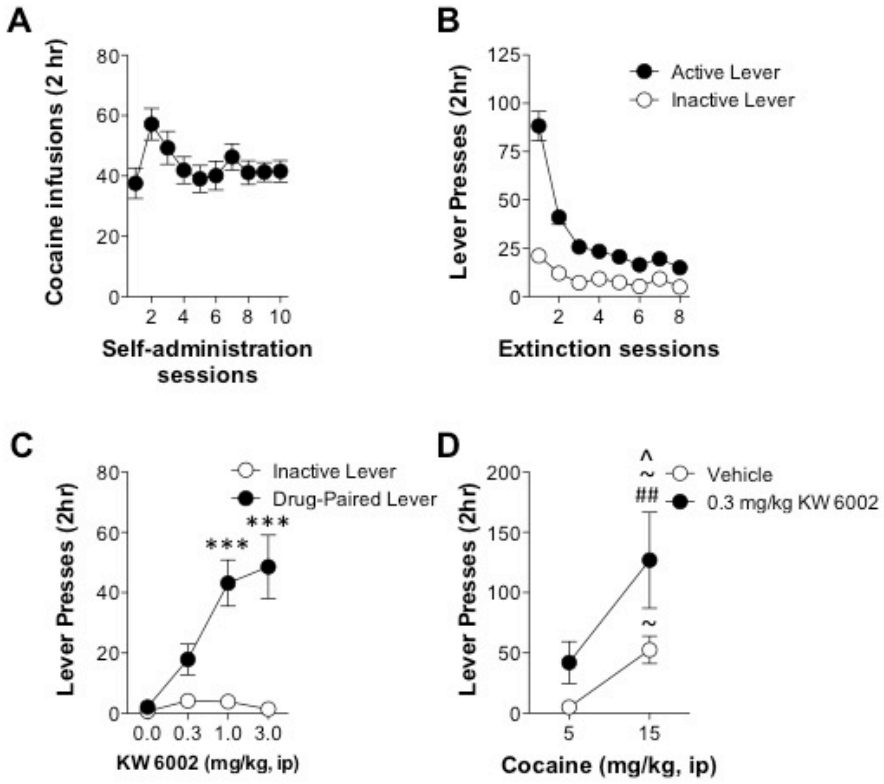

Figure 3.1 Postsynaptic A_{2A} receptor antagonism produces reinstatement and enhances cocaine-induced reinstatement. A) Animals self-administered cocaine in over the course of 10 consecutive days in 2-hr sessions. B) Following 24hrs of forced abstinence in the home cage animals underwent 8 consecutive days of extinction training in 2-hr sessions. C) Systemic administration of KW6002 produces cocaine seeking in a dose dependent manner. Right) Pretreatment with KW6002 increases reinstatement responding induced by 15 mg/kg of cocaine. *** $p< 0.001$ Indicates significant from vehicle; ~ $p<0.05$ Indicates significant from vehicle--5 mg/kg cocaine; ## $p< 0.01$ Indicates significant from vehicle--15 mg/kg cocaine; ^ $p<0.01$ Indicates significant from KW 6002--5 mg/kg cocaine

were no significant effects of KW 6002 or cocaine administration on inactive lever responding.

Postsynaptic Adenosine A_{2A} Antagonism Increases Cocaine Seeking Induced by Cortical Stimulation

Because we found that postsynaptic adenosine A_{2A} receptor blockade produced reinstatement and exacerbated cocaine-primed reinstatement, we decided to examine its effects on reinstatement induced by mPFC stimulation. Animals in these experiments had an average intake of 43.8 ± 3.7 daily cocaine infusions for the last 5 days of self-administration (data not shown). Intra-mPFC infusion of AMPA produced reinstatement that was enhanced by pretreatment with KW 6002 (Figure 3.2a). A significant treatment X lever interaction was observed ($F_{3,42}$=17.07; p<0.0001), and significant main effects of lever ($F_{1,42}$=78.35; p<0.0001) and treatment ($F_{3,42}$=21.39; p<0.0001). Bonferroni's posttests revealed that intra-mPFC AMPA infusion significantly increased active lever responding compared to vehicle (t_{42}=7.15; p<0.001) and KW 6002 alone (t_{42}=4.15, p<0.001), and pretreatment with KW 6002 exacerbated this effect (veh/Intra-PFC AMPA: t_{42}=4.51, p<0.001; veh/KW 6002: t_{42}=7.08, p<0.001; veh: t_{42}=9.58, p<0.001). There were no significant differences in inactive lever presses.

Similarly, local microinjections of cocaine in the PFC produced robust increases in active lever presses, which were exacerbated by postsynaptic adenosine A_{2A} receptor antagonism (Figure 3.2b). A significant treatment X lever interaction ($F_{3,42}$=9.93; p<0.0001), and significant main effects of lever ($F_{1,42}$=44.63; p<0.0001) and treatment

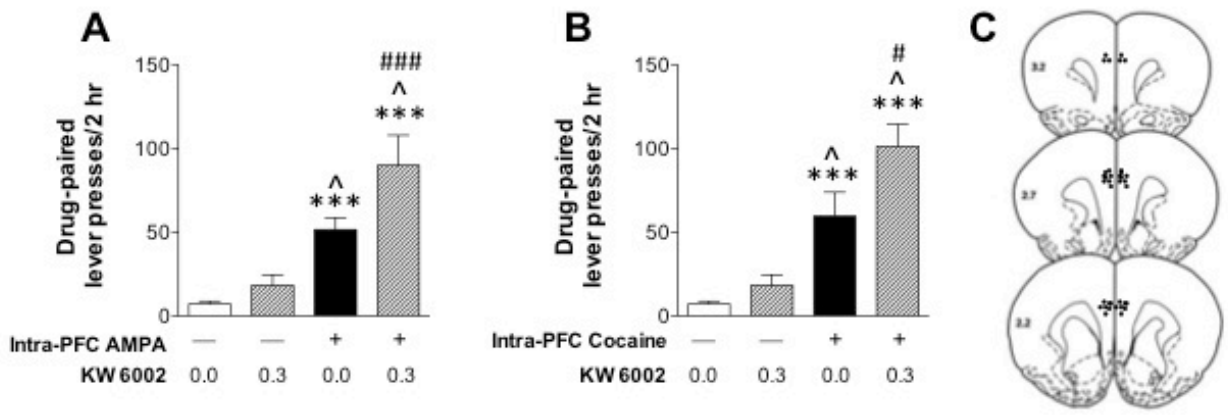

Figure 3.2 Effects of postsynaptic A_{2A} receptor blockade on reinstatement induced by prefrontal cortex stimulation. A) Intra-mPFC AMPA induces reinstatement which is enhanced by a pretreatment of KW 6002. B) Intra-mPFC cocaine also produces reinstatement which is amplified prior KW 6002 administration C) Microinjection sites of animals included in the data set. *** $p < 0.001$ Indicates significant from vehicle-vehicle; ^ $p < 0.001$ Indicates significant from KW 6002-vehicle; ### $p<0.001$ Indicates significant from vehicle--Intra-PFC AMPA; # $p<0.05$ Indicates significant from vehicle--Intra-PFC AMPA

($F_{3,42}$=14.40; p<0.0001) were found. Post hoc testing found that intra-mPFC administration of cocaine increased active lever presses compared to vehicle (t_{42}=6.35, p<0.001) and KW 6002 (t_{42}=3.86, p<0.001) treated animals, and treatment with KW 6002 before intra-mPFC cocaine administration resulted in amplified reinstatement responding compared to cocaine alone (t_{42}=2.54; p<0.05), KW 6002 alone (t_{42}=5.23, p<0.001), and vehicle (t_{42}=7.06, p<0.001). There were no significant effects of treatment on the inactive lever.

Postsynaptic Adenosine A_{2A} Antagonism Increases Cocaine Seeking Induced by Nucleus Accumbens Stimulation

Stimulation of dopamine receptors or AMPA receptors in the NAc shell can produce reinstatement (Bachtell *et al*, 2005; Cornish *et al*, 2000). Blockade of postsynaptic adenosine A_{2A} receptors in the nucleus accumbens shell has been shown to enhance cocaine seeking stimulated by systemic administration of cocaine (O'Neill *et al*, 2012). In this experiment we induced reinstatement with local injections of AMPA into the NAc shell to examine whether postsynaptic adenosine A_{2A} receptor antagonism would intensify intra-NAc AMPA-primed cocaine seeking. Animals in these experiments self-administered cocaine for 10 consecutive days and had an average intake of 39.4 ± 4.1 daily cocaine infusions for the last 5 days of self-administration (data not shown). Intra-NAc administration of AMPA following 2 hours of extinction increased active lever pressing, and this effect was enhanced by a pretreatment of KW 6002 (figure 3.3a). Analysis revealed a significant treatment X lever interaction ($F_{2,31}$=47.23, p<0.0001)

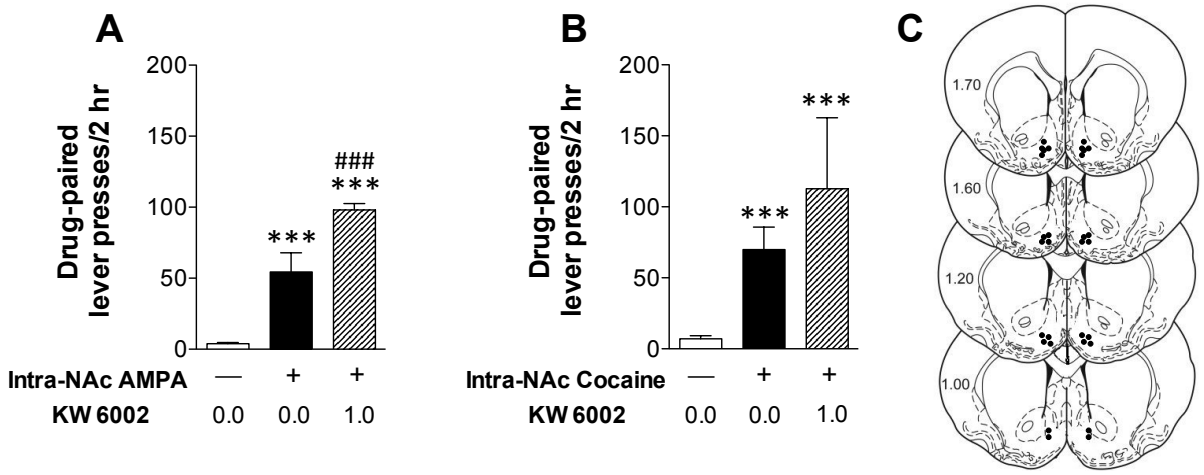

Figure 3.3 Effects of postsynaptic A_{2A} receptor blockade on reinstatement induced by nucleus accumbens stimulation. A) Intra-NAc AMPA produces reinstatement that is augmented by KW 6002. B) Intra-NAc cocaine also produces reinstatement. C) Microinjection sites of animals included in the data set. *** $p < 0.001$ Indicates significant from vehicle-vehicle; ### $p < 0.001$ Indicates significant from vehicle--Intra-NAc AMPA

and significant main effects of treatment ($F_{2,31}$=52.03, p<0.0001) and lever ($F_{1,31}$=171.1, p<0.0001). Bonferroni's posttests indicated that administration of AMPA into the NAc shell induced reinstatement compared to vehicle (t_{31}=8.23, p<0.001), and that blockade of postsynaptic A_{2A} receptors heightened cocaine seeking compared to vehicle (t_{31}=13.80, p<0.001) and intra-NAc AMPA alone (t_{31}=5.75, p<0.001). Responses on inactive levers were not affected by treatment.

Additionally, we found that both KW 6002 and vehicle treatments followed by local microinjections of cocaine into the nucleus accumbens shell initiated cocaine seeking in extinguished animals (figure 3.3b). A significant treatment X lever interaction ($F_{2,28}$=12.70, p<0.0001), and significant main effects of treatment ($F_{2,28}$=12.80, p<0.0001) and lever ($F_{1,28}$=53.53, p<0.0001) were found. Subsequent analysis uncovered a significant increased in reinstatement responding in animals treated with intra-NAc cocaine compared to vehicle treated animals (t_{28}=5.72, p<0.001). Pretreatment with the postsynaptic adenosine A_{2A} antagonist prior to intra-NAc cocaine infusion also produced cocaine seeking in comparison with vehicle treated animals (t_{28}=5.87, p<0.001). There were no significant effects of treatment on inactive lever presses.

Presynaptic Adenosine A_{2A} Receptor Antagonism Decreases Cocaine Seeking

Blockade of presynaptic adenosine A_{2A} receptors during extinction training has been shown to decrease subsequent cocaine seeking induced by cocaine (O'Neill *et al*, 2014). This suggests that antagonism of presynaptic adenosine A_{2A} receptors may have

opposing effects to postsynaptic adenosine A_{2A} receptor antagonism, which has been shown to increases cocaine related behaviors (Filip *et al*, 2006; O'Neill *et al*, 2012). Because systemic administration of KW 6002 produces dose-dependent increases in cocaine seeking, we first examined the effect of systemic injection of SCH 442416 on animals extinguished from cocaine self-administration. Animals in this experiment had an average intake of 51.9 ± 5.9 infusions of cocaine for the last 5 days of cocaine self-administration (data not shown). Administration of SCH 442416 at both 1.0 mg/kg and 3.0 mg/kg had no effect on active lever pressing (Figure 3.4a), however we did observe a significant main effect of lever ($F_{1,24}$=13.05, $p<0.01$) indicating that animals in all groups pressed the active lever significantly more than the inactive lever.

Next we examined the effects of SCH 442416 on cocaine-primed reinstatement. Animals in this experiment averaged 42.3 ± 4.9 cocaine infusions over the last 5 cocaine self-administration sessions (data not shown). Systemic cocaine injection (15 mg/kg) produced cocaine seeking which was blunted by a pretreatment with SCH 442416 (1.0 mg/kg) (Figure 3.4b). Statistical analysis revealed a significant treatment X lever interaction ($F_{2,21}$=24.25, $p<0.0001$) and significant main effects of treatment ($F_{2,21}$=24.02, $p<0.0001$) and lever ($F_{1,21}$=71.42, $p<0.0001$). Post hoc tests indicated that 15 mg/kg cocaine increased active lever presses compared to vehicle (t_{21}=9.78, $p<0.001$). Pretreatment with SCH 442416 prior to 15 mg/kg of cocaine significantly decreased active lever presses compared with cocaine alone (t_{21}=5.61, $p<0.001$), but active lever responding of this group was still significantly increased compared to vehicle (t_{21}=4.17, $p<0.001$). No differences in inactive lever pressing were observed.

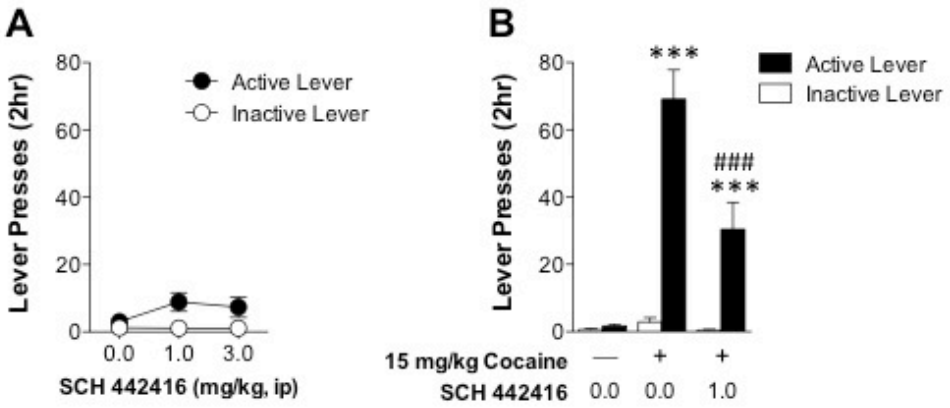

Figure 3.4 Presynaptic A_{2A} receptor antagonism has no effect on cocaine seeking and blunts cocaine-induced reinstatement. A) Systemic administration of SCH 442416 has no effect on cocaine seeking. B) Pretreatment with SCH 442416 blunts cocaine-primed reinstatement. *** $p < 0.001$ Indicates significant from vehicle-vehicle; ### $p < 0.001$ Indicates significant from vehicle-15 mg/kg cocaine

Presynaptic Adenosine A_{2A} Receptor Antagonism Blocks Cocaine Seeking Induced by Cortical Stimulation

Systemic administration of the presynaptic adenosine A_{2A} receptor antagonist, SCH 442416, has been shown to decrease glutamate release in the striatum induced by electrical stimulation of the PFC (Orru *et al*, 2011a). Because increased in glutamate release in the nucleus accumbens has been linked to reinstatement (McFarland *et al*, 2003) we examined the effect of SCH 442416 on reinstatement induced by prefrontal cortical stimulation. In these experiments, animals self-administered cocaine for 10 consecutive days (average intake for last 5 sessions: 50.6 ± 5.9). Intra-mPFC AMPA, again, produced reinstatement that was blocked by pretreatment with SCH 442416 (figure 3.5a). A significant treatment X lever interaction ($F_{2,41}$=23.83, $p<0.0001$) and significant main effects of treatment ($F_{2,41}$=19.48, $p<0.0001$) and lever ($F_{1,41}$=47.72, $p<0.0001$) were found. Bonferroni's posttests indicated that local microinjections of AMPA significantly increased responding on the active lever compared to vehicle (t_{41}=8.46, $p<0.001$), pretreatment with SCH 442416 abolished this effect (t_{41}=7.61, $p<0.001$). Inactive lever responding was not affected by any of the treatments.

Similarly, intra-mPFC cocaine induced reinstatement that was eliminated by blockade of presynaptic adenosine A_{2A} receptors (figure 3.5b). Analysis showed a significant treatment X lever interaction ($F_{2,41}$=9.18, $p<0.001$) and significant main effects of treatment ($F_{2,41}$=10.91, $p<0.001$) and lever ($F_{1,41}$=25.60, $p<0.0001$). Subsequent post hos tests revealed increased cocaine seeking following intra-mPFC cocaine compared to vehicle (t_{41}=6.06, $p<0.001$), and administration of SCH 442416

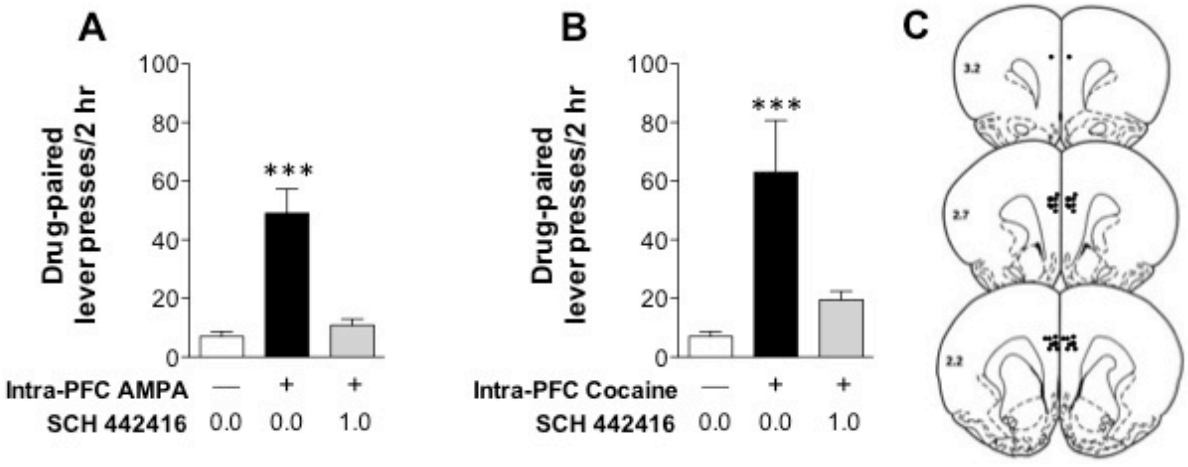

Figure 3.5 Effects of presynaptic A_{2A} receptor blockade on reinstatement induced by prefrontal cortex stimulation. A) Intra-mPFC AMPA induces cocaine seeking that is blocked by SCH 442416. B) Intra-mPFC cocaine also induces cocaine seeking. Again, pretreatment with SCH 442416 attenuates cocaine seeking. C) Microinjection sites of animals included in the data set. *** $p < 0.001$ Indicates significant from vehicle-vehicle

prior to intra-mPFC cocaine significantly decreased cocaine seeking compared to intra-mPFC cocaine alone (t_{41}=4.66, p<0.001). Lever presses on the inactive lever were not significantly different.

Presynaptic Adenosine A_{2A} Receptor Antagonism has no Effect on Cocaine Seeking Induced by Nucleus Accumbens Stimulation

Administration of AMPA or cocaine into the nucleus accumbens shell induces cocaine seeking, presumably by activating postsynaptic glutamate and dopamine receptors, respectively. We hypothesized that blockade of presynaptic adenosine A_{2A} receptors would not affect reinstatement mediated by direct stimulation of postsynaptic glutamate or dopamine receptors. In these experiments animals averaged 39.4 ± 4.1 cocaine infusions over the last 5 days of self-administration. As before, intra-NAc AMPA increased active lever presses, but pretreatment with the presynaptic adenosine A_{2A} receptors antagonist did not decrease reinstatement (figure 3.6a). A significant treatment X lever interaction ($F_{2,42}$= 20.15, p<0.0001) and significant main effects of treatment ($F_{2,42}$=17.27, p<0.0001) and lever ($F_{1,42}$=88.36, p<0.0001) were observed. Post hoc testing revealed that both intra-NAc AMPA alone (t_{42}=8.25, p<0.001) and pretreatment with SCH 442416 prior to intra-NAc AMPA infusion (t_{42}=6.17, p<0.001) significantly increased cocaine seeking compared to vehicle. No effects of treatment were found on inactive lever responding.

Similarly, intra-NAc cocaine infusion increased responding on the active lever, but pretreatment with SCH 442416 failed to reduce reinstatement (figure 3.6b). Analysis

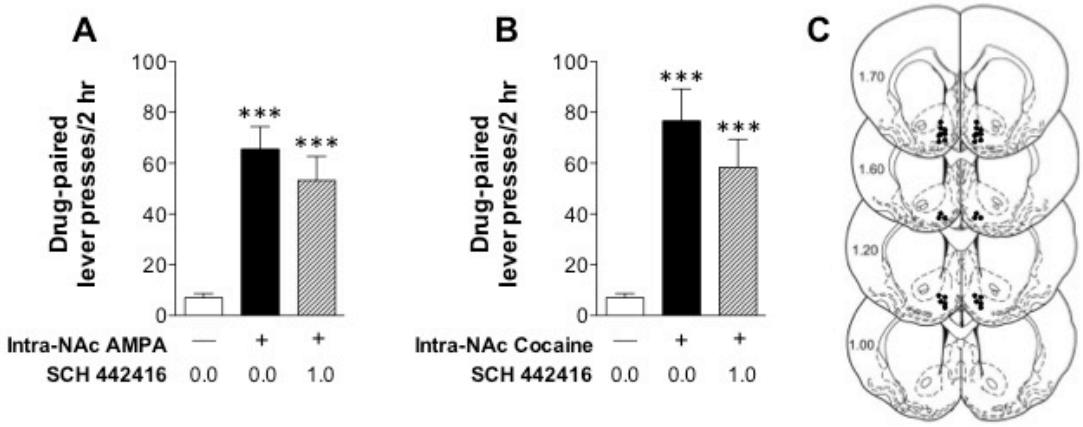

Figure 3.6 Effects of presynaptic A_{2A} receptor blockade on reinstatement induced by intra-NAc stimulation. A) Intra-NAc AMPA produces cocaine seeking, and pretreatment with SCH 442416 has no effect. B) Intra-NAc cocaine also induces reinstatement that is not altered by pretreatment with SCH 442416 C) Microinjection sites of animals included in the data set. *** $p < 0.001$ Indicates significant from vehicle-vehicle

revealed a significant treatment X lever interaction ($F_{2,39}$=11.80, p<0.0001) and significant main effects of treatment ($F_{2,39}$=17.57, p<0.0001) and lever ($F_{1,39}$=52.65, p<0.0001). Bonferroni's posttests showed that both SCH 442416 followed by intra-NAc cocaine infusion (t_{39}=5.71, p<0.001) and intra-NAc cocaine infusion alone (t_{39}=7.11, p<0.001) significantly increased reinstatement responding compared with vehicle. No effect of treatment on inactive lever responding was observed.

Discussion

The results of the studies presented here show that blockade of postsynaptic or presynaptic adenosine A_{2A} receptors produces opposing effects on cocaine seeking. We observed a dose dependent increase in cocaine seeking to KW 6002 alone, which has previously been described as an exclusively postsynaptic adenosine A_{2A} receptor antagonist (Orru *et al*, 2011a). We also found that pretreatment with KW 6002 enhanced cocaine-primed reinstatement. Similarly, cocaine seeking induced by AMPA or cocaine infusion into the mPFC or by AMPA infusion into the NAc was augmented by pretreatment with KW 6002.

These results are supported by previous literature showing that adenosine A_{2A} receptors exert tonic inhibition over dopamine D_2 receptors (Farrar *et al*, 2010; Hakansson *et al*, 2006; Harper *et al*, 2006), and confirms previous work indicating that blockade of adenosine A_{2A} receptors in the NAcc can facilitate cocaine seeking (O'Neill *et al*, 2012). As previously mentioned, adenosine A_{2A} receptors have their densest expression in the striatum and are highly co-localized with dopamine D_2 receptors on

striatopallidal MSNs (Fink *et al*, 1992; Svenningsson *et al*, 1999b) where they have opposing intracellular cascades. Stimulation of dopamine D_2 receptors in the NAc is necessary for cocaine-primed reinstatement (Anderson *et al*, 2006; Bachtell *et al*, 2005; Schmidt *et al*, 2006a), and decreasing inhibition from adenosine A_{2A} receptors amplifies signaling through these receptors (Filip *et al*, 2006; Hakansson *et al*, 2006; Harper *et al*, 2006). For example, blockade of adenosine A_{2A} receptors with KW 6002 prevents the effort-related behavioral deficits induced by a dopamine D_2 receptor antagonist (Nunes *et al*, 2010) and mimics the cellular signaling effects of a quinpirole, a dopamine D_2 receptor agonist (Hakansson *et al*, 2006).

Reinstatement responding was robustly enhanced by postsynaptic adenosine A_{2A} receptor blockade in nearly all studies measuring cocaine seeking with the notable exception of cocaine seeking induced by cocaine delivery into the NAc. This suggests that inhibition of postsynaptic adenosine A_{2A} receptors, presumably located on the indirect pathway MSNs, increases cocaine seeking regardless of whether dopamine or glutamate initiates reinstatement. Although the enhancement of reinstatement with pretreatment of KW 6002 was not seen with cocaine infusion into the NAc, we suspect there may have been a ceiling effect given that cocaine in the NAc with or without KW 6002 pretreatment produced robust cocaine seeking.

Interestingly, presynaptic adenosine A_{2A} receptor blockade had no effect on cocaine seeking alone, but blunted systemic cocaine-primed reinstatement. Additionally, infusion of either AMPA or cocaine into the mPFC induced reinstatement that was eliminated by presynaptic adenosine A_{2A} receptor blockade. However, intra-NAc AMPA

and cocaine induced cocaine seeking was unaffected by pretreatment with SCH 442416, the presynaptic adenosine A_{2A} receptor antagonist. This suggests that inhibition of presynaptic adenosine A_{2A} receptors located on glutamate terminals inhibits cocaine seeking presumably through a negative regulation of glutamate signaling from the mPFC.

Human studies have indicated that cocaine craving is associated with increased activity of the PFC (Kilts *et al*, 2001; Volkow *et al*, 1999a). Similarly, animal cocaine self-administration studies have suggested that glutamate signaling from the mPFC to the nucleus accumbens is necessary for cocaine-primed reinstatement (McFarland *et al*, 2003; Park *et al*, 2002; Rebec and Sun, 2005). Dopamine receptor stimulation, via intracranial infusion of cocaine, in the mPFC increases cortical glutamate release to the NAc and also plays a necessary role in cocaine-primed reinstatement (Park *et al*, 2002). Thus in our experiments, cocaine seeking induced by a mPFC infusion of cocaine, and likely AMPA, is dependent on glutamate release in the NAc. Previous studies have shown that antagonism of presynaptic adenosine A_{2A} receptors is able to decrease glutamate release into the striatum (Orru *et al*, 2011a) and if this is the case in the NAc, SCH 442416 is likely preventing glutamate release driven by mPFC stimulation. This explanation is further supported by the fact that reinstatement driven by intra-NAcc infusion of AMPA or cocaine, which is not dependent on mPFC glutamate release, was not decreased (affected) by blockade of presynaptic adenosine A_{2A} receptors with SCH 442416.

It is possible that our effects are due to blockade of adenosine A_{2A} receptors expressed outside of the striatum since administration of KW 6002 and SCH 442416 was systemic. However, this seems unlikely given the fact that adenosine A_{2A} receptors are mainly expressed in the striatum, and that previous work from our lab has shown intra-NAc antagonism of postsynaptic adenosine A_{2A} receptors produces increases in cocaine seeking (O'Neill *et al*, 2012). Additionally, systemic administration of SCH 442416 but not KW6002 decreases glutamate release in the striatum in response to electrical stimulation of cortex (Orru *et al*, 2011a). Furthermore, genetic deletion studies support the idea that conditional knockout of forebrain adenosine A_{2A} receptors, which would include presynaptic adenosine A_{2A} receptors, decrease sensitivity to psychostimulants (Bastia *et al*, 2005; Shen *et al*, 2008), while a conditional knockout of striatal adenosine A_{2A} receptors enhances sensitivity to psychostimulants (Shen *et al*, 2008).

Although it is not clear why SCH 442416 binds only presynaptic A_{2A} receptors, in vitro binding assays show that SCH 442416 has a decreased affinity for adenosine A_{2A} receptors when cells are co-transfected with dopamine D_2 receptors. Thus, it appears that the presence of dopamine D_2 receptors inhibits it's ability to block adenosine A_{2A} receptors, possibly due to the formation of A_{2A}-D_2 heteromeric receptor complexes (Orru *et al*, 2011a). This is not the case for the postsynaptic adenosine A_{2A} receptor antagonist, KW 6002 (Orru *et al*, 2011a). Because dopamine D_2 receptors and adenosine A_{2A} receptors are highly colocalized on MSNs of the indirect pathway within the striatum (Fink *et al*, 1992; Svenningsson *et al*, 1999b), SCH 442416 is unlikely to

have any direct effect on signaling within these neurons. Despite the unidentified mechanism for the binding of SCH 442416 to presynaptic adenosine A_{2A} receptors and KW 6002 to postsynaptic adenosine A_{2A} receptors, the opposing effects on cocaine seeking confirm that these antagonists are targeting different populations of adenosine A_{2A} receptors.

Together these findings support previous studies that revealed differential effects of pre- and postsynaptic adenosine A_{2A} receptor antagonism (O'Neill *et al*, 2014; Orru *et al*, 2011b). This work also validates previous findings indicating that adenosine receptors modulate cocaine related behaviors (Bachtell *et al*, 2009; Ferre *et al*, 2007; Hobson *et al*, 2012; Hobson *et al*, 2013; O'Neill *et al*, 2012) and may be a viable target for future pharmacotherapies. These findings are novel because they reveal the effects of adenosine A_{2A} receptor blockade on reinstatement mediated by mPFC activation in comparison with cocaine seeking induced in the NAc itself. Future studies should examine the cellular mechanisms that underlie the bi-directional effects of presynaptic compared to postsynaptic adenosine A_{2A} receptor blockade.

Chapter 4: Persistent reduction of cocaine seeking by pharmacological manipulation of adenosine A_1 and A_{2A} receptors during extinction training in rats

Abstract

Adenosine receptor stimulation and blockade have been shown to modulate a variety of cocaine-related behaviors. These studies identify the direct effects of adenosine receptor stimulation on cocaine seeking during extinction training and the persistent effects on subsequent reinstatement to cocaine seeking. Rats self-administered cocaine on a fixed ratio one schedule in daily sessions over 3 weeks. Following a 1-week withdrawal, the direct effects of adenosine receptor modulation were tested by administering the adenosine A_1 receptor agonist, N6-cyclopentyladenosine (CPA, 0.03 and 0.1 mg/kg), the adenosine A_{2A} agonist, CGS 21680 (0.03 and 0.1 mg/kg), the presynaptic adenosine A_{2A} receptor antagonist, SCH 442416 (0.3, 1, and 3 mg/kg), or vehicle prior to each of six daily extinction sessions. The persistent effects of adenosine receptor modulation during extinction training were subsequently tested on reinstatement to cocaine seeking induced by cues, cocaine, and the dopamine D_2 receptor agonist, quinpirole. All doses of CPA and CGS 21680 impaired initial extinction responding; however, only CPA treatment during extinction produced persistent impairment in subsequent cocaine- and quinpirole-induced seeking. Dissociating CPA treatment from extinction did not alter extinction responding or subsequent reinstatement. Administration of SCH 442416 had no direct effects on extinction responding but produced dose-dependent persistent impairment of cocaine- and

quinpirole-induced seeking. These findings demonstrate that adenosine A_1 or A_{2A} receptor stimulation directly impair extinction responding. Interestingly, adenosine A_1 receptor stimulation or presynaptic adenosine A_{2A} receptor blockade during extinction produces lasting changes in relapse susceptibility.

Introduction

Chronic cocaine use alters the signaling of numerous neurotransmitters throughout the brain, and these changes are thought to underlie the compulsive drug seeking that characterizes addiction (Koob and Volkow, 2010). Addicts are susceptible to relapse even after prolonged abstinence from cocaine, suggesting that drug-induced changes persist and contribute to drug relapse (Nestler, 2001). Relapse is modeled in rodents using the drug self-administration/reinstatement paradigm where rats are initially trained to perform an operant response to acquire a drug reinforcer. Rats then undergo extinction training resulting in newly learned contextual relationships, culminating in progressive decreases in responding in the previously drug-associated context (Bouton, 2004). Following extinction training, reinstatement can be induced by a priming injection of the previously self-administered drug, stress, or the reintroduction of a drug-associated cue (Shalev *et al*, 2002). This model has been used to identify pharmacotherapies that directly reduce reinstatement of drug seeking (Schmidt *et al*, 2010; Shalev *et al*, 2002; Uys and LaLumiere, 2008). However, recent studies have begun examining the effects of pharmacotherapies administered during abstinence or extinction training with the goal of finding treatments that produce enduring reductions in relapse susceptibility (Reichel *et al*, 2011; Zhou and Kalivas, 2008).

Adenosine is a ubiquitous neuromodulator that influences a variety of neurotransmitters through its activity at two adenosine receptor subtypes expressed in the brain. Adenosine A_1 and adenosine A_{2A} receptors are G protein-coupled receptors that produce inhibitory and stimulatory cellular effects, respectively (Svenningsson *et al*,

1999b). Recent work from our lab and others has demonstrated that both adenosine A_1 receptors and adenosine A_{2A} receptors are capable of modulating numerous cocaine-related behaviors. For example, stimulation of both adenosine A_1 receptors and adenosine A_{2A} receptors either systemically or in the nucleus accumbens blocks the expression of cocaine sensitization (Filip *et al*, 2006; Hobson *et al*, 2012). Blockade of adenosine A_{2A} receptors, on the other hand, enhances both expression and development of cocaine sensitization (Filip *et al*, 2006; Hobson *et al*, 2012). Similarly, stimulation of either adenosine A_1 receptors or adenosine A_{2A} receptors blocks the expression of cocaine conditioned place preference (Poleszak *et al*, 2002a). In a self-administration paradigm adenosine A_{2A} receptor stimulation attenuates acquisition of cocaine self-administration (Knapp *et al*, 2001), while antagonism enhances responding for cocaine on a progressive ratio schedule of reinforcement (Doyle *et al*, 2012). Finally, stimulation of both adenosine A1 receptors and adenosine A_{2A} receptors suppresses cocaine reinstatement, while blockade of adenosine A_{2A} receptors enhances cocaine seeking (Bachtell *et al*, 2009; Hobson *et al*, 2013; O'Neill *et al*, 2012).

In this study, we expand upon the role of adenosine receptors in cocaine seeking by stimulating adenosine receptor subtypes during extinction training. We assess the direct effect of adenosine A_1 receptor or adenosine A_{2A} receptor stimulation on cocaine seeking during extinction and the persistent effects on subsequent reinstatement induced by cocaine-associated cues and drug priming. We hypothesize that targeting adenosine receptors during the extinction phase of the self-administration/reinstatement

model will enhance the effects of extinction and produce lasting inhibitory effects on relapse susceptibility.

Materials and Methods

Animals and housing conditions

Male Sprague–Dawley rats (Charles River, Wilmington, MA) initially weighing 250–300 g were individually housed with food and water available *ad libitum*. All experiments were conducted during the light period of a 12 hr light/dark cycle in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the University of Colorado at Boulder.

Drugs

The adenosine A_{2A} receptor agonist, CGS 21680, was purchased from Tocris Bioscience (Ellisville, MO). The adenosine A_1 receptor agonist, CPA (N^6-cyclopentyladenosine), presynaptic adenosine A_{2A} receptor antagonist, SCH 442416(2-(2-Furanyl)-7-[3-(4-methoxyphenyl)propyl]-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine), dopamine D_2 receptor agonist, quinpirole ((–)-quinpirole hydrochloride), and cocaine hydrochloride were obtained from Sigma-Aldrich (St Louis, MO). All drugs, except SCH 442416, were dissolved in sterile-filtered physiological (0.9%) saline. The presynaptic A2AR antagonist, SCH 442416, was dissolved in 33% DMSO and 66% dd$H_2$0.

Cocaine Self-Administration Procedure

Self-administration procedures were performed in operant conditioning chambers (Med-Associates, St Albans, VT) equipped with two response levers and an infusion pump system. Animals were initially trained to lever press for sucrose pellets to facilitate acquisition of cocaine self-administration (O'Neill *et al*, 2012). After lever-press training, animals were fed *ad libitum* for at least 24 hr before catheter implantation surgery, and for the duration of the study. Surgery and cocaine self-administration procedures were similar to those described in O'Neill et al, 2012. Rats were implanted with jugular catheters under halothane anesthesia (1–2.5%). Rats were allowed 3–7 days recovery in their home cage before experimental procedures began. During this time, catheters were flushed daily with 0.1 ml heparinized saline. Animals showing signs of post-surgical distress were administered (*S*)-(+)-ketoprofen (5 mg/kg, s.c.), a non-steroidal anti-inflammatory analgesic (Carabaza *et al*, 1996). After recovery, animals were allowed to self-administer intravenous cocaine (0.5 mg/kg/100 µl injection) on an FR1 reinforcement schedule in daily 4 h sessions for 5–7 days per week. Cocaine injections were delivered over 5 s concurrent with the illumination of a cue light above the active lever and were followed by a 15 s time-out period (TO 20 s) when the house light remained off and responding produced no consequence. Inactive lever responses produced no consequence throughout testing. After a minimum of 15 cocaine self-administration sessions, animals remained in their home cages for 6 days of forced abstinence.

Effects of adenosine receptor stimulation and blockade on extinction responding

On days 7–12 following self-administration, animals returned to the operant conditioning chambers for 6 daily 4 h extinction training. Responses on the lever previously paired with cocaine injections during self-administration (active lever) and on the inactive lever were recorded, but had no programmed drug or cue delivery. The effect of adenosine receptor stimulation on responding on both levers was tested in animals counterbalanced for cocaine intake. Five minutes prior to the initiation of the extinction sessions, animals were treated with vehicle, the adenosine A_1 receptor agonist, (CPA: 0.03 mg/kg or 0.1 mg/kg, ip), the adenosine A_{2A} receptor agonist, (CGS 21680: 0.03 mg/kg or 0.1 mg/kg, ip), or the presynaptic adenosine A_{2A} receptor antagonist (SCH 442416: 0.3 mg/kg, 1 mg/kg or 3 mg/kg, ip). Doses of the adenosine agonists/antagonist were chosen based on previous findings illustrating their effects on lever responding in the absence of locomotor suppression or generalized effects on operant behavior (Bachtell *et al*, 2009; Hobson *et al*, 2012; Orru *et al*, 2011a).

Effects of temporally dissociating adenosine A_1 receptor stimulation from extinction training

In order to determine whether the effects of adenosine A_1 receptor stimulation during extinction on subsequent reinstatement are due to an enhancement of extinction learning we decided to temporally dissociate the adenosine A_1 receptor receptor stimulation from extinction training. This way we can assess the necessity of extinction training in the effectiveness of the adenosine A_1 receptor agonist in decreasing

subsequent reinstatement. All cocaine self-administration and extinction procedures were the same except that animals were treated with vehicle or CPA (0.1 mg/kg, ip) 4 h after the end of each 4 h extinction session with the first treatment administered 4 h after the first extinction session. We chose to administer the adenosine A_1 receptor agonist 4 h post extinction based on the pharmacokinetics of CPA (Mathot *et al*, 1993) and other experiments examining similar effects (Hammond *et al*, 2012; Mickley *et al*, 2012).

Reinstatement procedures

The enduring effects of the adenosine agonist/antagonist treatments administered during extinction training were also tested on reinstatement responding over the subsequent 4 days following extinction training using a repeated testing paradigm. Thus, all animals were tested for cue-, cocaine-, and dopamine D_2 agonist-induced reinstatement. All reinstatement tests consisted of a 4 h reinstatement session comprised of a 2 h of extinction phase followed by a 2 h reinstatement test phase. Cue-induced reinstatement was initiated by 5 non-contingent saline infusions paired with the illumination of the cocaine cue light (5 s) administered every 2 min over the first 10 min of the reinstatement phase. Throughout the reinstatement phase, responding at the previously active lever resulted in a 5 s cue light illumination and saline infusion. Responding at the inactive lever resulted in no cue or infusion. Cocaine-induced reinstatement was initiated by a systemic injection of cocaine (15 mg/kg, i.p.) 5 min prior to the reinstatement phase. Dopamine D_2 agonist-induced reinstatement was initiated

by a systemic injection of quinpirole (0.3 mg/kg, s.c.) 5 min prior to the reinstatement phase. Quinpirole was used to induce reinstatement because previous studies have shown that dopamine D_2 receptors play a key role in mediating cocaine-related behaviors (Bachtell *et al*, 2005; De Vries *et al*, 2002; Fontana *et al*, 1993; Graham *et al*, 2007; Khroyan *et al*, 2000; Merritt and Bachtell, 2013; Self *et al*, 1996). Thus, dopamine D_2 receptor stimulation produces behavioral cross-sensitization in animals receiving repeated cocaine and produces robust reinstatement responding in animals extinguished from cocaine self-administration. Responding at both levers was recorded, but resulted in no cues or reward delivery.

Data Analyses

The numbers of animals in each experimental group ranged from 6 to 22. Extinction data for animals receiving systemic injections of either adenosine A_1 receptor agonist or adenosine A_{2A} receptor agonist 5 min prior to extinction training were analyzed by a two-way mixed-design ANOVA with lever and treatments (vehicle, adenosine A_1 receptor agonist or adenosine A_{2A} receptor agonist) as the factors. Extinction data for animals receiving systemic injections of the presynaptic adenosine A_{2A} receptor antagonist 5 min prior to extinction training was analyzed by a separate two-way mixed-design ANOVA with lever and treatments (vehicle or adenosine A_{2A} receptor antagonist) as the factors. Lever responding during reinstatement testing was analyzed by a two-way ANOVA with session (extinction or reinstatement) and extinction treatments serving as the factors. Responding on the active and inactive levers (see Supplemental Results)

during reinstatement was analyzed separately by a two-way ANOVA. Significant interactions were followed with simple main effects analyses (one-way ANOVA) and *post hoc* tests (Bonferroni's comparisons). Statistical significance was set at $p < 0.05$ for all tests.

Results

Adenosine A_1 and A_{2A} receptor stimulation decreases initial extinction responding

Prior to extinction training, animals were assigned to treatment groups based on their cocaine intake over the last five self-administration sessions (Fig. 1a). Figure 1b illustrates that pretreatment with either CPA or CGS 21680 significantly decreased extinction responding on the first of six daily 4-h extinction training sessions. We observed a significant treatment X day interaction ($F_{20,280} = 1.70$, $p < 0.05$) and significant main effects of treatment ($F_{4,280} = 2.91$, $p < 0.05$) and day ($F_{5,280} = 38.94$, $p < 0.0001$). Subsequent analysis of the interaction revealed that pretreatment with CPA (0.3 and 0.1 mg/kg) and CGS 21680 (0.03 and 0.1 mg/kg) significantly reduced active lever responding compared to vehicle during the first extinction training session. Post hoc analysis revealed a significant reduction in lever responding of all treatment groups compared to vehicle (0.03 mg/kg CPA: $t_{280} = 4.14$, $p < 0.001$; 0.1 mg/kg CPA: $t_{280} = 4.38$, $p < 0.001$; 0.03 mg/kg CGS 21680: $t_{280} = 2.92$, $p < 0.05$; 0.1 mg/kg CGS 21680: $t_{280} = 4.05$, $p < 0.001$). Analysis of the first and second hour of active lever responding in the first extinction training session revealed a significant treatment X hour interaction ($F_{2,43} = 6.37$; $p = 0.0038$) and significant main effects of treatment ($F_{2,43} =$

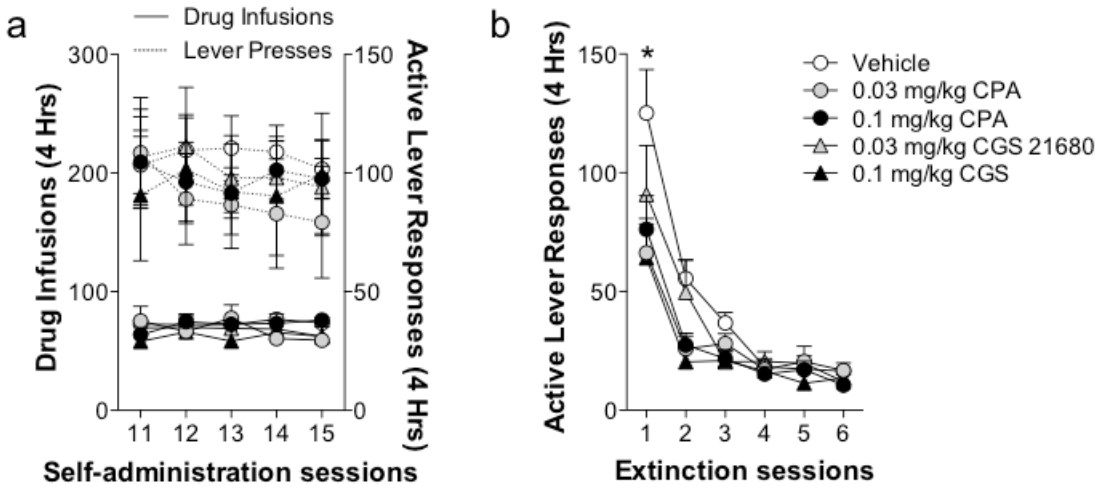

Figure 4.1 Stimulating adenosine A_1 or adenosine A_{2A} receptors decreases extinction responding during the first extinction session. a) Average number of cocaine infusions and active lever responses for each treatment group in each 4-h session over the last 6 days of the self-administration phase. b) Systemic administration of the adenosine A_1 receptor agonist, CPA or the A_{2A} receptor agonist, CGS 21860, reduced extinction responding on the first day of extinction training. Asterisk indicates significant from vehicle pretreatment (t test, $p < 0.05$)

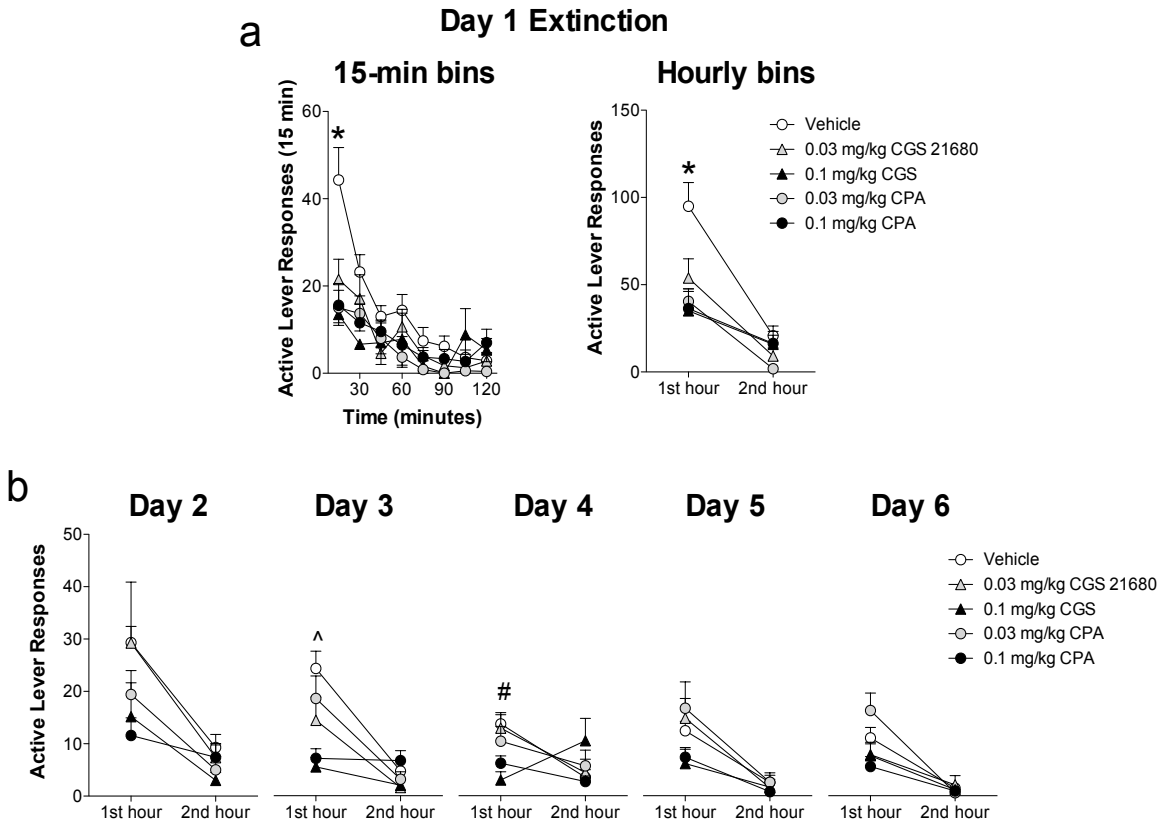

Figure 4.2 Temporal effects of stimulating adenosine A_1 or adenosine A_{2A} receptors during extinction training a) Systemic administration of the adenosine A_1 receptor agonist, CPA (0.03 and 0.1 mg/kg, i.p.) or the adenosine A_{2A} receptor agonist, CGS 21680 (0.03 and 0.1 mg/kg, i.p.), immediately prior to extinction significantly decreases active lever responses during the first 15 min of the first extinction session (left). Simple main effects analysis of the significant interaction found that a pretreatment with CPA 0.03 mg/kg ($t_{392} = 5.03$, $p < 0.001$), 0.1 mg/kg ($t_{392} = 6.26$, $p<0.001$), and CGS 21680 0.03 mg/kg ($t_{392} = 4.74$, $p < 0.001$), 0.1 mg/kg ($t_{392} = 4.98$, $p<0.001$) decreased active lever responding in the first 15 min of the first extinction session compared with vehicle control. During the 1st h of the first extinction session animals pretreated with either 0.03 mg/kg ($t_{56} = 3.15$, $p < 0.05$), 0.1 mg/kg ($t_{56} = 3.58$, $p < 0.01$) CGS 21680 or 0.03 mg/kg ($t_{56} = 3.45$, $p<0.01$) 0.1 mg/kg ($t_{56} = 4.71$; $p < 0.001$) CPA showed significantly reduced active lever pressing compared to a vehicle pretreatment (right). * Indicates all groups significant from vehicle pretreatment (t-test, $p < 0.05$) b) Administration of the adenosine A_1 or adenosine A_{2A} receptor agonist also has time-dependent effects throughout extinction training. Administration of 0.03 mg/kg and 0.1 mg/kg of the adenosine A_1 receptor agonist, CGS 21680 or 0.1 mg/kg of the adenosine A_1 receptor agonist CPA decreased extinction responding in the first hour of the 3rd extinction session (^ Significant from vehicle, t-test, $p < 0.05$). On the 4th day of extinction training 0.1 mg/kg CGS 21680 and 0.1 mg/kg CPA significantly decreased active lever responses in the first hour of the extinction session (# Significant from vehicle pretreatment, t-test, $p < 0.05$).

Table 4.1 Statistical analyses of adenosine agonist effects during the first 2 hours of extinction sessions 1-6

Extinction Session	Statistical Analysis
1	Hour: $F_{1,56} = 48.53$, $p < 0.0001$ Treatment: $F_{4,56} = 3.76$, $p < 0.01$ Interaction: $F_{4,56} = 5.07$, $p < 0.01$
2	Hour: $F_{1,56} = 19.77$, $p < 0.0001$ Treatment: NS Interaction: NS
3	Hour: $F_{1,56} = 25.77$, $p = 0.0001$ Treatment: $F_{4,56} = 4.05$, $p < 0.01$ Interaction: $F_{4,56} = 4.49$, $p < 0.01$
4	Hour: $F_{1,56} = 4.66$, $p < 0.05$ Treatment: NS Interaction: $F_{4,56} = 2.61$, $p < 0.05$
5	Hour: $F_{1,56} = 45.64$, $p < 0.0001$ Treatment: NS Interaction: NS
6	Hour: $F_{1,56} = 40.96$, $p < 0.0001$ Treatment: NS Interaction: NS

4.48; $p < 0.0171$) and hour ($F_{1,43} = 52.59$; $p < 0.0001$). We also analyzed responding during extinction training sessions 2 through 6 in hourly intervals over the first 2 h of each extinction session (figure 4.2b) Statistical analyses are presented in Table 4.1.

Adenosine A_1 and A_{2A} receptor stimulation decreases initial extinction responding at the previously inactive lever

Analyses of inactive lever responding during extinction training revealed significant main effects of day (2 h: $F_{5, 215} = 12.45$; $p < 0.0001$ & 4 h: $F_{5, 215} = 14.09$; $p < 0.0001$) and treatment in 2 h sessions ($F_{2, 215} = 3.76$; $p = 0.03$), but not in 4 h sessions ($F_{2, 215} = 1.58$, $p = 0.22$). No significant interactive effects were observed (2 h: $F_{10, 215} = 1.34$; $p = 0.21$ & 4 h: $F_{10, 215} = 1.14$, $p = 0.33$).

Further analysis of inactive lever responding in 15 m intervals of the first extinction session found a significant main effect of time ($F_{7, 301} = 6.19$; $p < 0.0001$), but not significant main effects of treatment ($F_{2, 301} = 1.83$; $p = 0.17$). No significant interactive effects were observed ($F_{14, 301} = 1.27$; $p = 0.22$). The hourly comparison of inactive lever responding revealed a significant main effect of hour in extinction sessions 1 ($F_{1,43} = 26.01$; $p < 0.0001$), 2 ($F_{1, 43} = 10.43$; $p < 0.01$), 3 ($F_{1, 43} = 5.06$; $p < 0.05$), 5 ($F_{1, 43} = 22.80$; $p < 0.0001$), and 6 ($F_{1, 43} = 19.16$; $p < 0.0001$), but not in extinction session 4 ($F_{1, 43} = 3.21$; $p = 0.08$). Significant main effects of treatment during the first 2 h of extinction session were observed in extinction sessions 2 ($F_{2, 43} = 4.09$; $p < 0.05$) and 4 ($F_{2, 43} = 4.24$; $p < 0.05$), but not in extinction sessions 1 ($F_{2, 43} = 1.88$; $p = 0.17$), 3 ($F_{2, 43} = 0.94$; $p = 0.40$), 5 ($F_{2, 43} = 0.07$; $p = 0.93$), or 6 ($F_{2, 43} = 1.37$; $p = 0.27$). No significant interactive

effects were observed in any of the extinction sessions (Session 1: $F_{2, 43} = 2.20$; $p = 0.12$; Session 2: $F_{2, 43} = 1.18$; $p = 0.32$; Session 3: $F_{2, 43} = 1.78$; $p = 0.18$; Session 4: $F_{2, 43} = 0.47$; $p = 0.63$; Session 5: $F_{2, 43} = 0.11$; $p = 0.89$; Session 6: $F_{2, 43} = 1.72$; $p = 0.19$).

Adenosine A_1 receptor stimulation during extinction training blunts subsequent cocaine- and quinpirole-induced reinstatement

We next assessed the persistent effects of adenosine receptor stimulation during extinction training on subsequent reinstatement testing. Figure 4.3 illustrates that the highest dose of CPA (0.1 mg/kg), but not the lower dose of CPA (0.03 mg/kg) or either dose of CGS 21680 administered during extinction training inhibited subsequent reinstatement induced by cocaine and quinpirole. None of the treatments had any effect on cue-induced reinstatement. Analysis of active lever responding during cue-induced reinstatement revealed a significant main effect of reinstatement for all animals (CPA experiment: $F_{1,39} = 72.56$, $p< 0.0001$; CGS experiment: $F_{1,36} = 69.59$, $p< 0.0001$). No treatment or treatment X reinstatement interaction effects were observed indicating that regardless of treatment during extinction, all animals reinstated similarly. Analysis of cocaine-induced reinstatement in animals treated with CPA during extinction training revealed a significant treatment X reinstatement interaction ($F_{2,39} = 3.63$, $p< 0.05$) and significant main effects of treatment ($F_{1,39} = 3.62$, $p< 0.05$) and reinstatement ($F_{1,39} = 36.17$, $p< 0.0001$). Subsequent analysis of the interaction revealed that rats treated with 0.1 mg/kg CPA during extinction showed reduced cocaine-induced reinstatement when compared to vehicle treated rats ($t_{39} = 3.76$, $p< 0.001$). Analysis of cocaine-induced

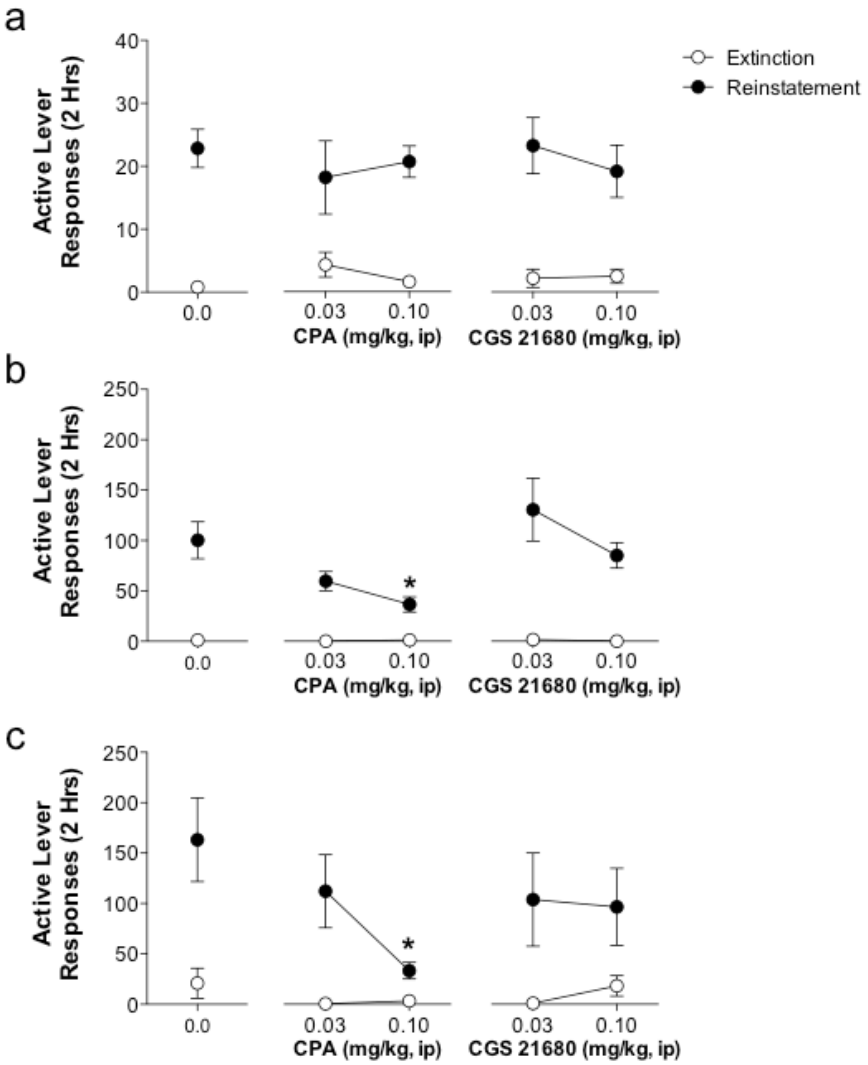

Figure 4.3 Persistent effects of stimulating adenosine A_1 receptors during extinction training on subsequent cocaine- and D_2-agonist-induced cocaine seeking. a) Pretreatment with the adenosine A_1 receptor agonist, CPA, or the adenosine A_{2A} receptor agonist, CGS 21680, during extinction training had no effect on subsequent cue-induced reinstatement. Subsequent reinstatement induced by b) cocaine and c) quinpirole was significantly blunted by administration of the adenosine A_1 receptor agonist, CPA (0.1 mg/kg), but not by the adenosine A_{2A} receptor agonist, CGS 21680, administered during extinction training. Asterisks indicate significant from vehicle pretreatment during extinction training (Bonferroni's post test, $p<0.001$)

reinstatement in animals treated with CGS 21680 during extinction revealed a significant main effect of reinstatement ($F_{1,36}$ = 42.78, p< 0.0001), but no main effect of treatment or treatment X reinstatement interaction. Analysis of quinpirole-induced reinstatement in animals treated with CPA during extinction training revealed a significant treatment X reinstatement interaction ($F_{2,37}$ = 3.56, p< 0.05) and significant main effects of treatment ($F_{1, 37}$ = 3.81, p< 0.05) and reinstatement ($F_{1, 37}$ = 18.84, p < 0.0001). Subsequent analysis of the interaction found that animals treated with 0.1 mg/kg CPA during extinction showed less D_2 agonist-induced reinstatement responding compared to vehicle animals (t_{37} = 3.80, p< 0.001). Analysis of quinpirole-induced reinstatement in animals treated with CGS during extinction showed a significant main effect of reinstatement ($F_{1,34}$ = 15.56, p< 0.001), but no main effect of treatment or treatment X reinstatement interaction.

Adenosine A_1 receptor stimulation temporally dissociated from extinction training has no effect on extinction responding or subsequent reinstatement responding

Given the persistent effects of CPA to diminish subsequent reinstatement, we next assessed whether adenosine A_1 receptor stimulation temporally dissociated from the extinction training sessions would recapitulate these effects. Animals were separated into balanced treatment groups based on cocaine intake prior to extinction training (figure 4.4). Four hours after the end of each extinction training session, animals were administered either vehicle or 0.1 mg/kg CPA, the dose effective in reducing subsequent reinstatement (see above). Analysis of extinction responding at the active

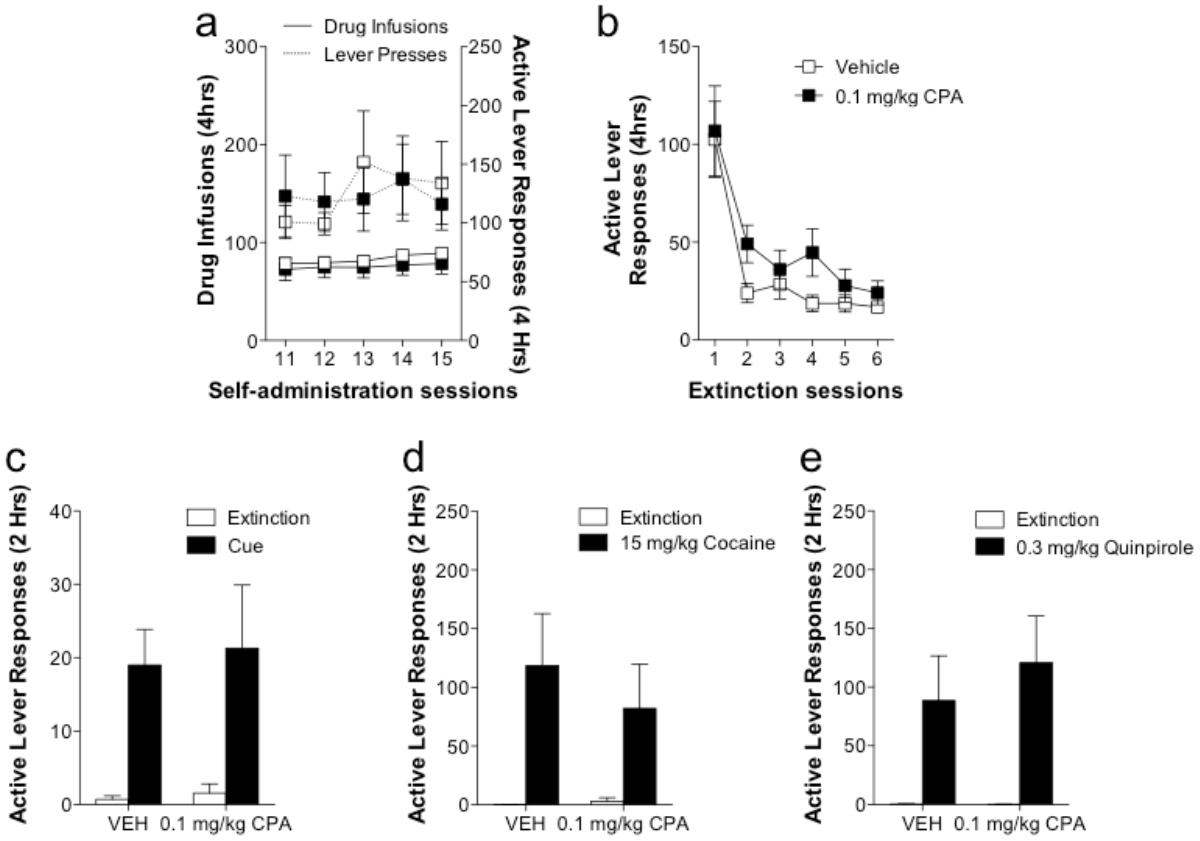

Figure 4.4 Dissociating adenosine A_1 receptor stimulation from extinction training has no effect on extinction responding or subsequent reinstatement. a) Average number of cocaine infusions and active lever responses in each 4-h session over the last 5 days of the self-administration phase. b) Systemic treatment with the adenosine A_1 receptor agonist, CPA (0.1 mg/kg, i.p.), 4 h after each extinction session has no effect on extinction responding. Adenosine A_1 receptor stimulation temporally dissociated from extinction training has no effect on c) cue-, d) cocaine-, or e) dopamine D_2-agonist-induced reinstatement.

lever revealed a significant main effect of session (F 5,60 = 24.49, p<0.0001), but there was no effect of treatment or the session X treatment interaction. Following extinction training, animals were tested for cue-, cocaine-, and quinpirole-induced drug seeking (figure 4.4). In all reinstatement tests, there was a significant main effect of reinstatement (cue: F 1,12 = 14.25, p<0.01; cocaine: F 1,12 = 11.46, p<0.01; quinpirole: F 1,12 = 14.27, p<0.01), but there was no significant treatment or treatment X reinstatement interaction suggesting that dissociating adenosine A_1 receptor stimulation from the extinction training sessions is not sufficient to produce these persistent effects on reinstatement.

Adenosine A_1 receptor stimulation temporally dissociated from extinction training has no effect on extinction responding or subsequent reinstatement responding at the previously inactive lever

Analysis of inactive lever responding during extinction training revealed no significant differences between treatment groups ($F_{1,60}$ = 1.84, p = 0.1998) or the treatment X session interaction ($F_{5,60}$ = 1.28, p = 0.2852), but a significant main effect of session was observed ($F_{1, 12}$ = 7.08; p< 0.0001).

Inactive lever responding was also evaluated and there were no significant main effects of session on cue- ($F_{2, 42}$ = 2.38; p = 0.10), or quinpirole-induced ($F_{2, 42}$ = 2.38; p = 0.10) reinstatement, however, analysis of cocaine-induced drug seeking revealed a significant main effect of session ($F_{1, 12}$ = 12.58; p = 0.004). No significant main effects of treatment were detected in cue- ($F_{1, 12}$ = 0.11; p = 0.75), cocaine- ($F_{1, 12}$ = 0.13; p =

0.91), or quinpirole-induced ($F_{1, 12} = 0.89$; $p = 0.36$) reinstatement. Additionally, no interactive effects were observed in any of the reinstatement tests (cue: $F_{1, 12} = 0.21$; $p = 0.66$; cocaine: $F_{1, 12} = 0.50$; $p = 0.49$; quinpirole: $F_{1, 12} = 0.89$; $p = 0.36$).

Adenosine A_{2A} receptor blockade has no effect on extinction responding

Prior to extinction training, animals were assigned to treatment groups based on their cocaine intake over the last five self-administration sessions (figure 4.5). Lever responding was then extinguished in six daily sessions where a pretreatment of vehicle or the adenosine A_{2A} receptor antagonist, SCH 442416 (0.3, 1, or 3 mg/kg) was administered prior to each extinction training session (figure 4.5). These doses of SCH 442416 were chosen based on previous work illustrating that low doses (0.3 and 1.0 mg/kg) primarily inhibit presynaptic adenosine A_{2A} receptors decreasing both locomotor activity and evoked glutamate release, while 3.0 mg/kg inhibits postsynaptic adenosine A_{2A} receptors to increase locomotor activity (Orru *et al*, 2011a; Orru *et al*, 2011b). Analysis of extinction responding over the entire 4-h session revealed a significant main effect of session (4 h: $F_{5,135} = 79.04$, $p < 0.0001$; 2 h: $F_{5,135} = 85.74$, $p < 0.0001$), but there was no main effect of treatment or treatment X session interaction.

Presynaptic adenosine A_{2A} receptor blockade has no effect on extinction responding at the previously inactive lever

Analysis of inactive lever responding during the extinction sessions revealed significant main effects of session (2 h: $F_{5, 70} = 4.79$; $p < 0.001$ & 4 h: $F_{5, 70} = 4.78$; $p <$

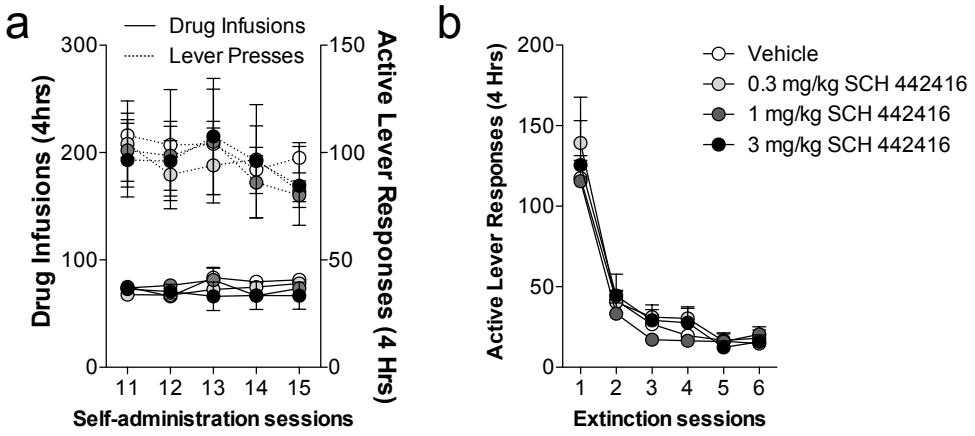

Figure 4.5 Blocking adenosine A_{2A} receptors during extinction has no effects on extinction responding. a) Average number of cocaine infusions and active lever responses in each 4-h session over the last 6 days of the self-administration phase. b) adenosine A_{2A} receptor antagonism by SCH 442416 (0.3, 1, or 3 mg/kg, i.p.) has no effect on extinction responding when administered immediately prior to the beginning of each 4-h extinction session

0.001). No significant main effects of treatment (4 h: $F_{1, 70} = 0.01$; $p = 0.95$) or significant interactive effects (4 h: $F_{5, 70} = 0.38$; $p = 0.89$) were observed on inactive lever responding during extinction.

Presynaptic A_{2A} receptor blockade during extinction training decreases subsequent cocaine- and quinpirole-induced reinstatements

SCH 442416 administered during extinction training dose dependently inhibited subsequent reinstatement induced by cocaine and quinpirole but had no effect on cue-induced reinstatement (figure 4.6). Analysis of active lever responding during cue-induced reinstatement revealed a significant main effect of reinstatement (F 1,26 = 58.12, $p < 0.0001$), but there was no main effect of treatment or treatment X reinstatement interaction. Analysis of active lever responding during cocaine-induced reinstatement revealed a significant treatment X reinstatement interaction (F 3,27 = 4.02, $p < 0.05$) and significant main effects of treatment (F 3,27 = 3.98, $p < 0.05$) and reinstatement (F 1,27 = 29.42, $p < 0.0001$). Post hoc analyses demonstrate that pretreatment with either 0.3 or 1.0 mg/kg SCH 442416 during extinction training significantly reduced cocaine-induced reinstatement compared to vehicle and 3 mg/kg SCH 442416 (vehicle vs 0.3 SCH 442416: t 27 = 2.40, $p < 0.05$, vehicle vs 1.0 SCH 442416: t 27 = 2.79, $p < 0.05$). Analysis of active lever responding during quinpirole-induced reinstatement revealed a significant treatment X reinstatement interaction (F 3,26 = 3.13, $p < 0.05$), and significant main effects of treatment (F 3,26 = 3.05, $p < 0.05$) and reinstatement (F 1,26 = 36.70, $p < 0.0001$) were observed. Post hoc analyses

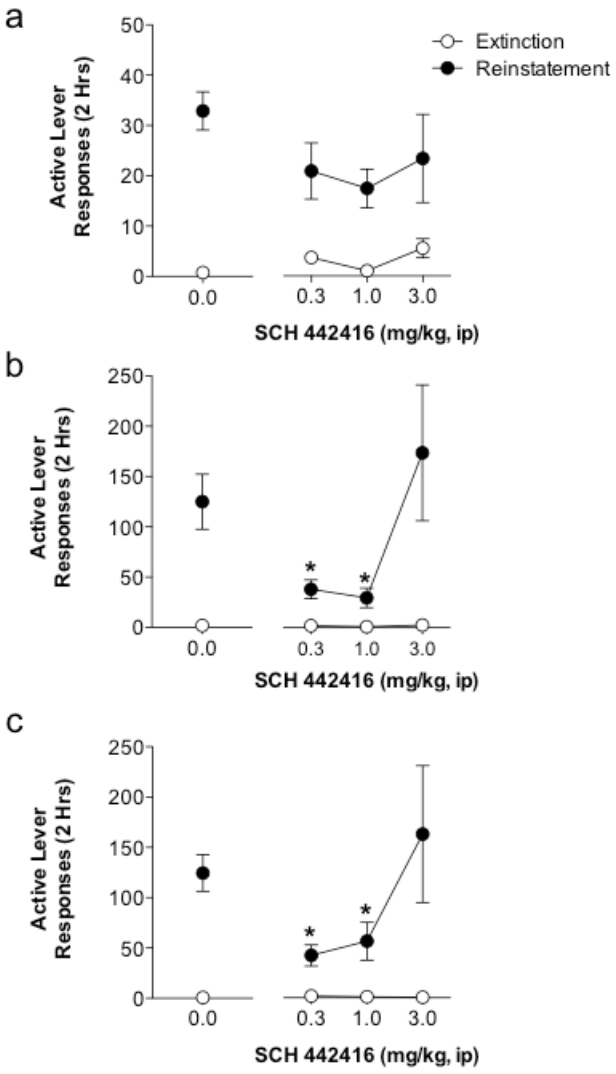

Figure 4.6 Blocking presynaptic, but not postsynaptic, adenosine A_{2A} receptors during extinction produces enduring reductions on reinstatement of cocaine seeking. a) Blocking adenosine A_{2A} receptors during extinction training has no effect on subsequent cue-induced reinstatement. b) Pretreatment of SCH 442416 during extinction training impaired subsequent reinstatement of cocaine-induced seeking when administered at doses effective at blocking presynaptic adenosine A_{2A} receptors (0.3 or 1 mg/kg). c) Similarly, antagonism of presynaptic adenosine A_{2A} receptors during extinction also impaired subsequent cocaine seeking induced by quinpirole. *Asterisks* indicate significant from vehicle pretreatment (*t* test, $p<0.05$)

demonstrate that pretreatment with either 0.3 or 1.0 mg/kg SCH 442416 during extinction training significantly reduced quinpirole-induced reinstatement compared to vehicle and 3 mg/kg SCH 442416 (vehicle vs 0.3 SCH 442416: $t\,27 = 2.72$, $p < 0.05$ and vehicle vs 1.0 SCH 442416: $t\,27 = 2.34$, $p < 0.05$).

Presynaptic A_{2A} receptor blockade during extinction decreases subsequent cue-, cocaine- and D_2 agonist-induced reinstatement at the previously inactive lever

Inactive lever responding from the reinstatement session was compared to inactive lever responding from the last hour of the extinction phase that occurred immediately prior. No significant main effects of session were observed for any of the reinstatement sessions (cue: $F_{1, 14} = 3.84$; $p = 0.07$; cocaine: $F_{1, 14} = 3.93$; $p = 0.07$; quinpirole: $F_{1, 14} = 2.05$; $p = 0.17$). For cue-induced reinstatement, a significant main effect of treatment ($F_{1,14} = 5.90$; $p < 0.05$) was observed, however, no significant main effect of treatment was detected for cocaine- ($F_{1, 14} = 0.11$; $p = 0.75$) or quinpirole-induced ($F_{1, 14} = 0.35$; $p = 0.56$) reinstatement. No interactive effects were observed (cue: $F_{1, 14} = 3.03$; $p = 0.10$; cocaine: $F_{1, 14} = 0.20$; $p = 0.66$; quinpirole: $F_{1, 14} = 0.35$; $p = 0.56$).

Discussion

We have previously shown that stimulation of adenosine receptors can directly attenuate the reinstatement of cocaine seeking induced by pharmacological stimuli (Bachtell *et al*, 2009; Hobson *et al*, 2013; O'Neill *et al*, 2012). Here, we examine the effect of adenosine receptor stimulation or blockade on extinction responding and

subsequent reinstatement. Our findings reveal that stimulation of adenosine A_1 receptors or adenosine A_{2A} receptors inhibit initial extinction responding, which parallels previous work from our lab and others illustrating that adenosine receptor stimulation can inhibit different types of cocaine seeking including the initiation of cocaine taking (Knapp *et al*, 2001), as well as cue- and drug- primed reinstatement (Bachtell *et al*, 2009; Hobson *et al*, 2013; O'Neill *et al*, 2012). We suspect these reductions in initial cocaine seeking observed on the first day of extinction training are due to the ability of stimulation at postsynaptic adenosine A_1 receptors and adenosine A_{2A} receptors to antagonize activity of dopamine D_1 and D_2 receptors, respectively (Franco *et al*, 2007; Fuxe *et al*, 2007a; Tozzi *et al*, 2007; Yabuuchi *et al*, 2006). However, only adenosine A_1 receptor stimulation produced lasting reductions in cocaine- and dopamine D_2 agonist-induced cocaine seeking. These persistent effects on reinstatement were not observed when adenosine A_1 receptor stimulation was temporally dissociated from extinction training. In order to further elucidate the role of adenosine receptors in cocaine seeking we examined the effects of antagonizing presynaptic adenosine A_{2A} receptors, a mechanism known to facilitate presynaptic adenosine A_1 receptor activity (Orru *et al*, 2011a), during extinction training. While this treatment had no direct effect on extinction responding, it produced persistent decreases in cocaine- and dopamine D_2 agonist-induced cocaine seeking. Notably, antagonism of postsynaptic adenosine A_{2A} receptors had no effect on extinction or subsequent reinstatement. These results suggest that adenosine modulation during extinction can produce lasting effects on reinstatement.

It is important to consider the anatomical and neuronal locations of adenosine receptors when interpreting our results. Both Adenosine A_1 receptors and adenosine A_{2A} receptors are highly localized to the NAc, caudate and putamen where they have been shown to regulate cocaine-mediated responses (Ferre *et al*, 2011; Fink *et al*, 1992; Fuxe *et al*, 2007a; Hobson *et al*, 2012; O'Neill *et al*, 2012). Presynaptic adenosine A_1 receptors in the striatum are located on glutamate terminals to reduce basal glutamate release (Corsi *et al*, 1997; Mahan *et al*, 1991; McCool and Farroni, 2001; Quarta *et al*, 2004; Solinas *et al*, 2002). Presynaptic adenosine A_{2A} receptors are expressed specifically on glutamate terminals that synapse onto dopamine D_1 receptor-expressing GABA neurons of the direct pathway where they act to enhance glutamate release (Corsi *et al*, 1997; Martire *et al*, 2011; Orru *et al*, 2011b; Quarta *et al*, 2004; Quiroz *et al*, 2009; Rosin *et al*, 1998; Sebastiao and Ribeiro, 1996). Postsynaptic adenosine A1 receptors are expressed on the direct pathway neurons of the striatum where they oppose actions of dopamine D_1 receptors and decrease glutamate receptor trafficking (Fuxe *et al*, 2007a; Hobson *et al*, 2013). Postsynaptic adenosine A_{2A} receptors colocalize with dopamine D_2 receptors on the indirect pathway striatal neurons where they oppose the intracellular signaling cascades of dopamine D_2 receptors and increase glutamate receptor trafficking (Fuxe *et al*, 2007a; Hakansson *et al*, 2006; Tozzi *et al*, 2007). Cocaine self-administration produces persistent alterations in glutamate homeostasis in the NAc and other reward-related brain areas (Baker *et al*, 2003a; Cornish *et al*, 2000; Kalivas *et al*, 2005; McFarland *et al*, 2003; Pierce *et al*, 1996; Reid and Berger, 1996). Cocaine seeking is associated with reduced basal extracellular

glutamate levels and increased release of glutamate in the NAc in response to a cocaine prime (McFarland *et al*, 2003). Thus, adenosine receptors in the striatum are capable of modulating both dopamine and glutamate signaling to impair cocaine seeking during extinction and reinstatement procedures. We suspect our effects are primarily mediated by adenosine receptors within the striatum, although future studies are necessary to identify the contribution of additional brain regions (see below).

Given the presynaptic and postsynaptic actions of adenosine A_1 receptors to reduce glutamate neurotransmission in the striatum, we were somewhat surprised to observe decreased cocaine seeking throughout extinction training. Previous evidence suggests that increased, not decreased, glutamate activity during extinction facilitates this new learning (Nic Dhonnchadha *et al*, 2010; Self *et al*, 2004; Sutton *et al*, 2003; Thanos *et al*, 2011a; Thanos *et al*, 2011b). However, adenosine A_1 receptors are expressed throughout the brain where they inhibit glutamate signaling (Mahan *et al*, 1991). It seems likely that stimulation of adenosine A_1 receptors in areas such as the hippocampus or basolateral amygdala are involved in decreasing extinction responding since both of these structures play a role in context-induced cocaine seeking (Cooper *et al*, 2006; Fuchs *et al*, 2005; Kalivas *et al*, 2003; Lasseter *et al*, 2010; Ramirez *et al*, 2009; Schmidt *et al*, 2005; Wells *et al*, 2013). Stimulation of adenosine A_1 receptors in either the hippocampus (Branisteanu *et al*, 1987; Poli *et al*, 1991) or basolateral amygdala (Heinbockel and Pape, 1999; McCool *et al*, 2001) inhibits the activity of these structures and we suspect that this may contribute to the direct effects decreased of adenosine A_1 receptor stimulation on extinction responding.

Adenosine A_1 receptor stimulation during extinction training also produced lasting decreases in cocaine- and dopamine D_2 agonist-induced reinstatement. The persistent effects on subsequent reinstatement testing may result from decreases in overall glutamate release in the NAc and other areas during extinction training since dissociation of adenosine A_1 receptor stimulation from extinction sessions did not produce the same lasting effects. This decreased glutamate release coupled with postsynaptic adenosine A_1 receptors that reduce glutamate signaling in direct pathways neurons may help to consolidate extinction-induced changes that impair subsequent reinstatement. We determined if presynaptic adenosine receptors played a preferential role in these persistent effects by administering several doses SCH 442416, a presynaptic adenosine A_{2A} receptor antagonist and facilitator of presynaptic adenosine A_1 receptors inhibitory actions on glutamate terminals, as demonstrated by its ability to reduce cortically-evoked glutamate release in the striatum (Orru *et al*, 2011a). We observed that presynaptic, but not postsynaptic antagonism of adenosine A_{2A} receptors during extinction produced lasting decreases on reinstatement, although it did not have any direct effects on extinction responding. This may be partly due to the selective presynaptic expression of adenosine A_{2A} receptors on cortical glutamate terminals onto direct pathway neurons (Orru *et al*, 2011a; Quiroz *et al*, 2009). Only two previous studies have identified and examined the presynaptic actions of SCH 442416 (Orru *et al*, 2011a; Orru *et al*, 2011b). This work provides more evidence for SCH 442416 as a presynaptic adenosine A_{2A} receptor antagonist at low doses since the high dose (3 mg/kg) of SCH 442416 had opposite effects on reinstatement compared to the 2 lower

doses (0.3 and 1 mg/kg). Together, these findings suggest that presynaptic adenosine A_1 receptor stimulation of cortical terminals may produce lasting effects on cocaine seeking when concurrent with extinction training, while postsynaptic adenosine A_1 receptor stimulation alone or in combination with presynaptic adenosine A_1 receptor stimulation reduces extinction responding directly.

Adenosine A_{2A} receptor stimulation resulted in decreased cocaine seeking during the first day of extinction training, but had no effect on subsequent reinstatement responding. This initial reduction in extinction responding is likely due to mild increases in overall glutamate transmission in the NAc during extinction. Increased glutamate transmission could result from either presynaptic adenosine A_{2A} receptor stimulation of glutamate terminals that synapse onto the direct pathway neurons (Corsi *et al*, 1997; Martire *et al*, 2011; Orru *et al*, 2011b; Sebastiao *et al*, 1996) or postsynaptic adenosine A_{2A} receptors that offset dopamine D2 receptor inhibition of glutamate signaling in the indirect pathway neurons (Ferre *et al*, 2011; Mayfield *et al*, 1993; Mingote *et al*, 2008; Shindou *et al*, 2003). These results are comparable to the facilitation of extinction observed with the partial NMDA glutamate receptor agonist, d-cycloserine (Thanos *et al*, 2011a; Thanos *et al*, 2011b). It is unclear why adenosine A_{2A} receptor stimulation does not have persistent effects akin to d-cycloserine (Paolone *et al*, 2009), especially because extinction appears to increase expression of adenosine A_{2A} receptors, and this alteration would likely lead to decreased relapse susceptibility (Frankowska *et al*, 2013). This may be due to adenosine A_{2A} receptor stimulation not effectively influencing

presynaptic glutamate transmission as we suspect occurs with presynaptic adenosine A_1 receptor stimulation or presynaptic adenosine A_{2A} receptor blockade.

It is possible that our adenosine agonists and antagonist are affecting astrocytic mechanisms of neurotransmitter release/reuptake, which could contribute to our behavioral effects. In addition to alterations in glutamate signaling, changes in GABA signaling have also been implicated in cocaine seeking (Filip and Frankowska, 2007a, 2008; Filip *et al*, 2007b; Frankowska *et al*, 2008a, b; Tang *et al*, 2005; Torregrossa *et al*, 2008; Wydra *et al*, 2013). In fact, cocaine self-administration appears to increase basal extracellular GABA in the accumbens and ventral pallidum (Wydra *et al*, 2013) and decrease GABAb receptor binding (Frankowska *et al*, 2008a, b). Cocaine-primed reinstatement results in increases in GABAb receptor binding (Frankowska *et al*, 2008a) and decreases extracellular GABA in the ventral pallidum (Tang *et al*, 2005; Torregrossa *et al*, 2008). Increasing adenosine transmission in the accumbens results in increased expression of glial glutamate transporter (GLT-1) mRNA and glutamate uptake (Wu *et al*, 2010), which is associated with persistent attenuation of cocaine- and cue-primed reinstatement (Knackstedt *et al*, 2010). It seems unlikely that our behavioral effects are due to increases in GLT-1 since blockade of adenosine A_{2A} receptors mimicked the effects of adenosine A_1 receptor stimulation on subsequent cocaine seeking. On the other hand, stimulation of adenosine A_1 receptors decreases GABA transport into astrocytes, while stimulation of adenosine A_{2A} receptors increase the uptake of GABA into astrocytes (Cristovao-Ferreira *et al*, 2013; Kirk and Richardson, 1994). Thus, increasing extracellular GABA through adenosine A_1 receptor stimulation

or adenosine A_{2A} receptor blockade could countermand the GABA decrease associated with reinstatement. It is currently unclear how chronic adenosine A_1 receptor stimulation or adenosine A_{2A} receptor blockade during extinction training may affect extracellular GABA levels either basally or in response to a pharmacological-prime. Future studies should investigate the role of adenosine receptor modulation on GABA transmission.

Further research is necessary to fully elucidate the role of adenosine receptors in extinction and subsequent reinstatement. All experiments presented here used systemic administration of adenosine receptor agonists and antagonists; microinjections targeting these receptors specifically in the NAc would clarify the contributions of adenosine receptors located elsewhere in the brain. Future studies should also use microdialysis to identify the effects of adenosine A_1 receptor and adenosine A_{2A} receptor stimulation as well as presynaptic adenosine A_{2A} receptor blockade on extracellular glutamate and GABA in the NAc during extinction and subsequent drug-primed reinstatement.

Together, these findings build upon evidence demonstrating that adenosine receptor stimulation negatively regulates cocaine seeking in a variety of situations. These findings are novel because they illustrate lasting effects of a pharmacological treatment administered during extinction training on drug-induced cocaine seeking. This type of phenomenon may provide the basis for realistic treatment of human cocaine addiction, where it is often not feasible to treat an acute relapse episode. Future studies should examine the mechanisms by which presynaptic adenosine A_1 receptor stimulation and/or presynaptic adenosine A_{2A} receptor blockade produces these lasting effects on cocaine seeking.

Chapter 5: General Discussion

The data presented here support recent findings that distinct populations of adenosine receptors within the striatum exist (Orru *et al*, 2011a; Orru *et al*, 2011b), and targeting these receptors can differentially modulate cocaine seeking following chronic self-administration. We suspect the ability for adenosine receptors to modulate cocaine seeking in these various ways is related to their modulatory effects on dopamine and glutamate signaling within the ventral striatum. However, because we have examined only pharmacological effects on behavior the cellular mechanisms that underlie these findings remain ambiguous and much of this discussion is speculative regarding the mechanisms explanations of these findings.

Mechanisms of Adenosine Signaling Within the Ventral Striatal Microcircuit

The experiments presented in Chapter 2 show the direct effects of adenosine A_{2A} receptor stimulation or blockade in the NAc on cocaine- or dopamine D_2 receptor agonist-induced reinstatement. Stimulation of adenosine A_{2A} receptors in the accumbens blocks cocaine- and quinpirole-induced drug seeking. However, at the same dose used in the cocaine reinstatement experiments, adenosine A_{2A} receptor agonism has no effect on cocaine-induced locomotor activity or sucrose reinstatement indicating the specificity of these effects on cocaine seeking. Systemic and intra-NAc blockade of adenosine A_{2A} receptors induces mild reinstatement, but antagonism of NAc adenosine A_{2A} receptors in combination with sub-threshold doses of cocaine and quinpirole exacerbates reinstatement.

The mechanisms underlying these effects are unclear, but it is possible that stimulation of the adenosine A_{2A} receptor facilitates the formation of A_{2A}-D_2 heteromers, ultimately decreasing ligand binding at dopamine D_2 receptors and restoring the behavioral changes following chronic cocaine administration. It remains unclear whether heteromeric A_{2A}-D_2 receptor complexes or another interactive mechanism mediate our effects since receptors that are not in heteromeric complexes still play an antagonistic and reciprocal role in modulating cellular function (Ferre, 1997; Ferre *et al*, 1991a). Thus, administration of an adenosine A_{2A} receptor agonist reverses the effects of a dopamine D_2 receptor agonist on intracellular Ca^{2+} release (Yang *et al*, 1995) and immediate early gene expression in the striatum (Morelli *et al*, 1994; Svenningsson *et al*, 1999a). Additionally, intracellular signaling cascades of adenosine A_{2A} and dopamine D_2 receptors (see figure 5.1) have opposite effects on cAMP production and neuronal excitability (Schiffmann *et al*, 2007; Svenningsson *et al*, 1999a; Tozzi *et al*, 2007). In fact a different study from our lab, involving adenosine A_1 receptor stimulation counteracting dopamine D_1 agonist-induced cocaine seeking, found that PKA-mediated phosphorylation of AMPA receptors plays an important role in regulating reinstatement (Hobson *et al*, 2012).

Stimulation of adenosine A_{2A} receptors activates enkephalin-containing neurons in the striatum, which form the indirect pathway (Karcz-Kubicha *et al*, 2006; Svenningsson *et al*, 1999a), while stimulation of dopamine D_2 receptors inhibits activity at these same neurons (Svenningsson *et al*, 1999a). Decreased GABA release in the ventral pallidum is associated with cocaine seeking (Tang *et al*, 2005), and

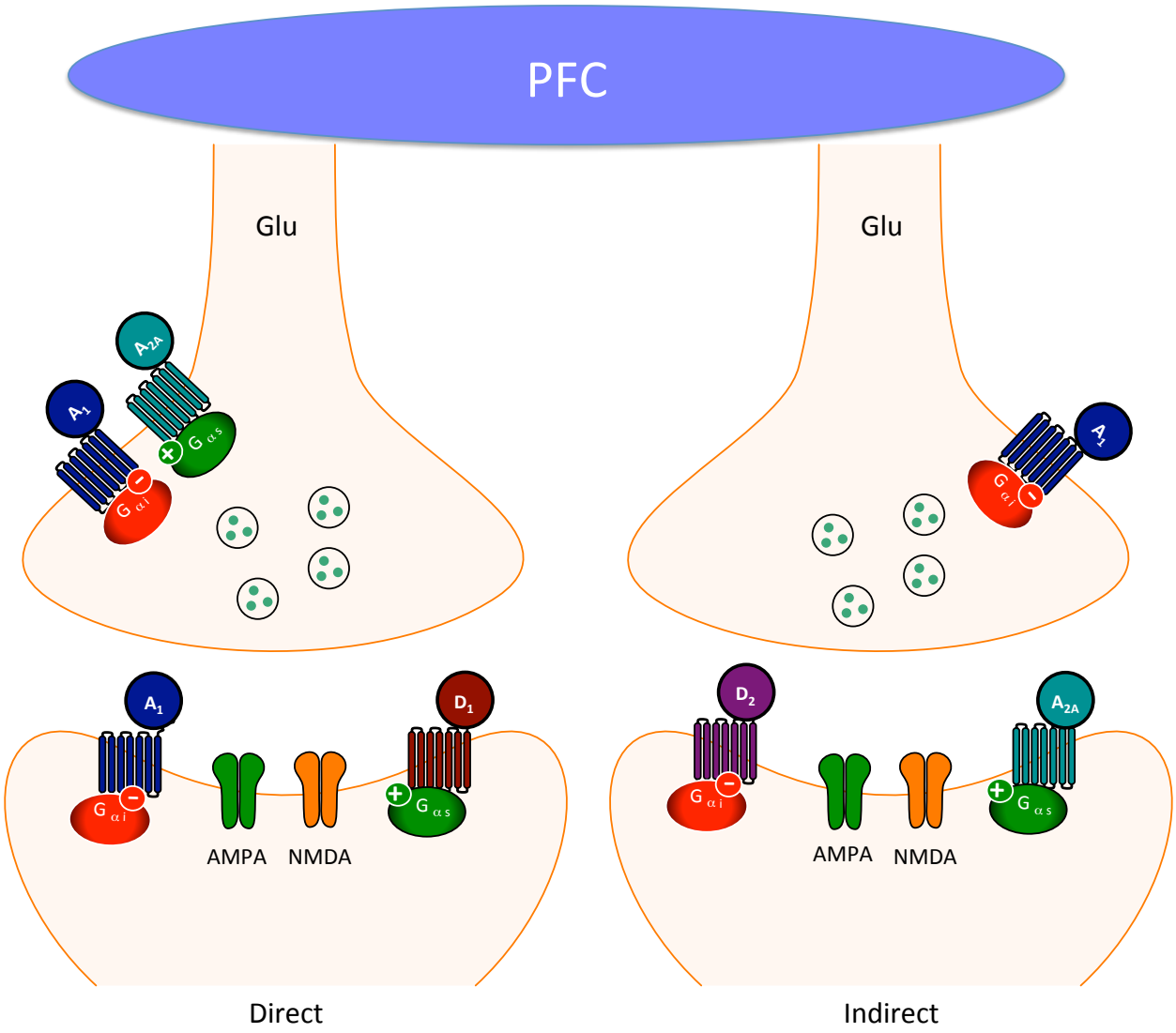

Figure 5.1 Populations of adenosine receptors in the NAc. Postsynaptic adenosine A_1 and dopamine D_1 receptors are colocalized on the direct pathway MSNs, while postsynaptic adenosine A_{2A} and dopamine D_2 receptors are colocalized on the indirect pathway. Presynaptic adenosine A_1 receptors are present on all glutamate terminals in the NAc, but presynaptic adenosine A_{2A} receptors are preferentially located on neurons that synapse onto the direct pathway.

dopamine D_2 receptor stimulation in the NAc results in decreased GABA in the ventral pallidum through the indirect pathway (Floran *et al*, 1997). Interestingly, stimulation of adenosine A_{2A} receptors in the ventral striatum results in enhanced GABA input to downstream structures like the ventral pallidum (Mingote *et al*, 2008; Ochi *et al*, 2000). Together, these findings suggest that the reduction in cocaine seeking seen with adenosine A_{2A} stimulation in the accumbens may be mediated by restoring cocaine-induced decreases in GABA release in the ventral pallidum. Similarly, blocking the tonic inhibition of adenosine A_{2A} receptors on dopamine D_2 receptors allows minor stimulation of dopamine D_2 receptors to further decrease GABA in the ventral pallidum and potentially drive cocaine seeking behaviors.

It is worth noting that the mild reinstatement seen with MSX-3, an adenosine A_{2A} receptor antagonist, may be related to combined actions at presynaptic and postsynaptic adenosine A_{2A} receptors. Our studies in Chapter 3 show that KW 6002, an antagonist thought to have greater specificity to postsynaptic adenosine A_{2A} receptors (Orru *et al*, 2011a), resulted in much more robust reinstatement responding when given alone. Also, administration of CGS 21680 possibly stimulates both pre- and postsynaptic adenosine A_{2A} receptors, but it's ability to inhibit dopamine D_2 receptor signaling and stimulate indirect pathway neurons appears to be capable of overcoming any increase in glutamate release mediated by presynaptic receptors and administration ultimately blocks cocaine seeking.

The experiments presented in chapter 3 examine the distinct effects of pre- and postsynaptic adenosine A_{2A} receptor blockade on cocaine seeking. For these

experiments we used KW 6002 and SCH 442416 because a previous study has shown that these compounds exhibit preferential post- and presynaptic profiles, respectively (Orru *et al*, 2011a). Similar to our previous data systemic administration KW 6002 produced strong cocaine seeking alone, and pretreatment also intensified reinstatement to a sub-threshold dose of cocaine. Conversely, systemic administration of SCH 442416 did not induce reinstatement on it's own, and pretreatment dampened cocaine-induced drug seeking. We suspect that the ability for SCH 442416 to reduce cocaine seeking is due to a decrease in glutamate release in the NAc, while the ability for KW 6002 to induce and enhance reinstatement is mostly likely due to removing the tonic inhibition of adenosine A_{2A} receptors on dopamine D_2 receptors.

In order to verify this, we induced reinstatement by infusing either AMPA or cocaine into the mPFC or the NAc. A previous study has shown that cocaine infusion into the mPFC induces reinstatement that can be attenuated by blocking AMPA receptors in the NAc (Park *et al*, 2002), presumably infusion of AMPA into the mPFC would also result in the increased glutamate release in the NAc that generates reinstatement (McFarland *et al*, 2004; Torregrossa *et al*, 2008). Infusion of AMPA into the NAc has been shown to induce reinstatement through actions at glutamate receptors on MSNs (Cornish *et al*, 1999; Cornish *et al*, 2000; Ping *et al*, 2008; Suto *et al*, 2004), while infusion of cocaine into the NAc produces reinstatement through stimulation of dopamine receptors on both the direct and indirect pathway (Bachtell *et al*, 2005; Schmidt *et al*, 2006b). As expected, pretreatment with KW 6002 exacerbated cocaine seeking induced by infusion of AMPA or cocaine into the mPFC. Surprisingly,

reinstatement responding to an intra-NAc infusion of AMPA, but not cocaine, was enhanced by pretreatment with KW 6002. Although this was unexpected, we may be observing a ceiling effect since infusing cocaine directly into the NAc results in high rates of responding on the previously drug-paired lever. It's also possible that allowing enhanced stimulation of dopamine D_2 receptors by adenosine A_{2A} receptor antagonism coupled with cocaine-induced dopamine increases shifted the animals into more stereotyped behavior since dopamine D_2 receptors are thought to be responsible for stereotypy following chronic psychostimulant use (Ujike *et al*, 1990). Nevertheless, this data suggests that KW 6002 does in fact act postsynaptically to enhance the inhibitory effects of dopamine D_2 receptors on indirect pathway neurons. Interestingly, pretreatment with SCH 442416 has no effect on reinstatement induced by intra-NAc infusion of AMPA or cocaine, but does block cocaine seeking stimulated by infusion of AMPA or cocaine into the mPFC. This effect supports the idea that blockade of presynaptic adenosine A_{2A} receptors decreases mPFC glutamate release into the NAc and consequently blocks cocaine seeking.

The differential effects of pre- and postsynaptic adenosine A_{2A} receptors on cocaine seeking is supported by a study showing that striatal-specific knockdown of A_{2A} receptors enhances locomotor activity in response to cocaine, while a forebrain-specific knockdown of adenosine A_{2A} receptors reduces cocaine-induced locomotor activity (Shen *et al*, 2008). The data presented here suggests that blockade of postsynaptic adenosine A_{2A} receptors located on MSNs in the NAc enhance cocaine seeking, while blockade of presynaptic adenosine A_{2A} receptors on glutamatergic terminals decreases

cocaine seeking. Taken together, these findings suggest that adenosine A_{2A} receptors localized on the indirect pathway provide inhibitory control over cocaine seeking, whereas adenosine A_{2A} receptors localized to glutamate terminals in the NAc enhance glutamate signaling to stimulate cocaine seeking.

The experiments presented in chapter 4 investigate the effects of adenosine receptor agonism and antagonism on extinction and subsequent reinstatement after chronic cocaine self-administration. Both adenosine A_1 and A_{2A} receptor agonists administered prior to extinction training decreased responding on the previously drug-paired lever on the first day of extinction training, however this effect was minor (confined to the first 15 min of the session) and was not seen in subsequent extinction training sessions. Remarkably, adenosine A_1 receptor stimulation during extinction training decreased subsequent reinstatement responding to cocaine and quinpirole, but if administration of the agonist was dissociated from extinction training no effects on subsequent reinstatement were observed. Given that reinstatement was inhibited by adenosine A_1, but not A_{2A}, receptor stimulation, we suspected that modulating glutamate signaling during extinction training was most likely mediating this effect. This is because in vivo microdialysis experiments have reported little to no change in dopamine release during extinction, but large increases in glutamate (Suto *et al*, 2010). In fact, a more recent study has shown that increases in accumbal glutamate during extinction training correlates with cocaine expectancy (Suto *et al*, 2013). Adenosine A_1 receptors are expressed on glutamate terminals in many parts of the brain, including the NAc, where stimulation will decrease glutamate release. Because presynaptic adenosine A_{2A}

receptor blockade, which had no effect on extinction training, also attenuated subsequent cocaine and quinpirole-induced drug seeking it seems likely that tempering glutamate release during extinction training may provide a way to reverse striatal signaling altered by chronic cocaine use. More importantly, this method of treatment represents a more viable option for many addicts due to its persistent benefits.

Based on the findings in the experiments presented here, we suspect that the direct and indirect pathways are playing distinctly different roles in cocaine reinstatement. Because dopamine D_1 receptors are stimulatory, chronic cocaine taking results in repeated activation of the direct pathway, while simultaneously exerting inhibitory actions through dopamine D_2 receptors resulting in repeated inactivation of the indirect pathway. This is supported by studies showing increased spine density in dopamine D_1 but not D_2 neurons following chronic cocaine exposure (Kim *et al*, 2011; Lee *et al*, 2006). Additionally, increases in mini EPSCs and decreases in mini IPSCs in direct pathway neurons coupled with decreases in mini EPSCs in indirect pathway neurons has been observed (Kim *et al*, 2011). Chronic cocaine has also been shown to increase ΔFosB in dopamine D_1, but not dopamine D_2 neurons (Lobo *et al*, 2013). Interestingly, overexpression of ΔFosB in the direct pathway, but not the indirect pathway, results in enhanced excitatory synaptic strength, spine density, and behavioral responses to cocaine (Grueter *et al*, 2013). Due to the opposing G protein coupling and intracellular signaling cascades of dopamine D_1 and D_2 receptors (Bertran-Gonzalez *et al*, 2008), chronic cocaine exposure may facilitate glutamate activation of dopamine D_1 neurons while inhibiting glutamate activation of dopamine D_2 neurons (Lobo *et al*, 2011).

MSNs of the striatum typically exhibit hyperpolarized resting membrane potentials (Cepeda *et al*, 2008; Planert *et al*, 2013). At baseline indirect pathway neurons are more excitable than direct pathway neurons, but dopamine modulates this excitability in an opposing fashion (Cepeda *et al*, 2008; Lobo *et al*, 2011; Planert *et al*, 2013). Dopamine increases intrinsic excitability in dopamine D_1 neurons, but decreases intrinsic excitability in dopamine D_2 neurons (Cepeda *et al*, 2008; Planert *et al*, 2013). Dopamine D_1 receptor stimulation enhances PKA activity and alters Ca^{2+} and K^+ channels to enhance the glutamate mediated "up-state" in these MSNs (Gerfen *et al*, 2011; Lobo *et al*, 2011; Surmeier *et al*, 2007). Conversely, dopamine D_2 receptor stimulation decreases PKA activity and alters Ca^{2+}, Na^+, and K^+ channels decreasing glutamatergic reactivity and shifting these MSNs into a “down-state” (Gerfen *et al*, 2011; Lobo *et al*, 2011; Surmeier *et al*, 2007). This imbalance in striatal signaling may ultimately underlie the vulnerability to relapse seen in chronic cocaine users. In fact, the circuitry of the indirect pathway is such that reduced activation results in decreased GABA release in the ventral pallidum which results in exacerbated inhibition of the medial dorsal thalamus and ultimately less activation of the PFC. Impaired signaling in this circuitry (Agnoli *et al*, 2013; Pezze *et al*, 2007) may explain the lack of behavioral control observed in cocaine addicts (Coffey *et al*, 2003; Garavan and Stout, 2005; Kaufman *et al*, 2003; Verdejo-Garcia *et al*, 2006).

Presynaptic adenosine A_{2A} receptors in the NAc are preferentially expressed on glutamate terminals that synapse on to dopamine D_1 expressing neurons (see figure 5.1) (Quiroz *et al*, 2009). They are therefore able to selectively modulate glutamate

release to the direct pathway but not the indirect pathway. This makes them an ideal target for decreasing glutamate signaling in dopamine D_1 neurons and possibly reversing imbalanced striatal signaling resulting from chronic cocaine use. We hypothesize that the long-term decreases in relapse susceptibility that we observe in the extinction experiments presented in chapter 4 are due to a reversal of this imbalance. Extinction training increases glutamate release in the NAc, but blocking presynaptic adenosine A_{2A} receptors decreases glutamate release at direct pathway synapses while leaving glutamate release to indirect pathway neurons unaffected. This is ideal because increased glutamate signaling to dopamine D_2 expressing neurons will likely increase the excitability of this chronically inhibited pathway, while simultaneously decreasing excitability in the over activated dopamine D_1 neurons. Together these effects may induce plasticity that approximate pre-cocaine conditions and restore neurotransmission in the ventral striatum, or at the very least help to decrease relapse vulnerability in addicts.

Potential Mechanisms of Adenosine Signaling in Glia

Adenosine A_1 and A_{2A} receptors are also expressed on astrocytes and microglia. It is possible that the behavioral effects we observe are in part due to actions of the adenosine agonists and antagonists on these cells that have been implicated in cocaine addiction (Beardsley and Hauser, 2014; Vijayaraghavan, 2009). Astrocytes are particularly important in regulating extracellular neurotransmitter levels, including glutamate. Importantly, because we know that glutamate homeostasis is disrupted by

chronic cocaine exposure adenosine actions at astrocytes may play an important role in decreasing reinstatement. Overexpression of adenosine A_1 receptors in astrocytes has been shown to increase expression and function of EAAT2 (excitatory amino acid transporter 2) also known as GLT-1 (Wu *et al*, 2011). EAAT2/GLT-1 is an important regulator of extracellular glutamate, and several studies have shown increasing expression with ceftriaxone, a β-lactam antibiotic, decreases cue and cocaine-induced reinstatement restores alterations in glutamate homeostasis (Knackstedt *et al*, 2010; Sari *et al*, 2009; Trantham-Davidson *et al*, 2012). Therefore, it is possible that adenosine A_1 receptor stimulation during extinction decreases subsequent reinstatement by increasing EAAT2/GLT-1 expression. However, because dissociation of adenosine A_1 receptor agonism from extinction training failed to decrease subsequent cocaine seeking this seems unlikely. Stimulation of adenosine A_{2A} receptors on astrocytes appears to inhibit EAAT2/GLT-1 mediated glutamate uptake, and prolonged activation of these receptors can decrease expression of these transporters (Matos *et al*, 2012; Nishizaki *et al*, 2002). If our presynaptic adenosine A_{2A} receptor antagonist is binding to astrocytic adenosine receptors, it is feasible that its ability to decrease subsequent reinstatement following administration during extinction training (chapter 4) or acutely decrease reinstatement (chapter 3) are related to increased glutamate uptake by astrocytes. Still, if this were the case we would expect that administration of SCH442416 would also decrease reinstatement induced by intra-NAcc AMPA or cocaine infusion, which it has no effect on.

Microglia have also been implicated in the addictive processes of cocaine and other psychostimulants (Beardsley *et al*, 2014; Northcutt *et al*, 2015). Increased

activation of microglia can increase extracellular glutamate as well as AMPA and NMDA receptor expression (Grace *et al*, 2014). The effects of adenosine receptor stimulation on microglia are complex. Adenosine A_1 receptor agonism decreases morphological activation of microglia (Luongo *et al*, 2014), while adenosine A_{2A} receptors appear to increase activation of microglia (Orr *et al*, 2009). However, at least one study has reported that instead of increasing proinflammatory cytokine release from these activated microglia, adenosine A_{2A} receptors act to inhibit release of proinflammatory cytokines, like TNF-α, induced by an LPS challenge (Newell *et al*, 2015). It is difficult to determine whether our effects on cocaine seeking are mediated by adenosine receptors on microglia since there is conflicting data regarding the effects of adenosine A_{2A} receptor stimulation on microglia exist, but it is plausible that decreasing extracellular glutamate through microglial mechanisms would decrease reinstatement.

Conclusions

Cocaine addiction is a significant public health problem, and the rate of relapse in addicts is very high. Identifying mechanisms to decrease relapse susceptibility is of utmost importance. Decades of research has identified that chronic cocaine use results in significant changes in glutamate and dopamine neurotransmission in NAcc, and that these nuanced changes are responsible for persistence of drug seeking behaviors even after prolonged abstinence. The data presented here suggest that adenosine, a known modulator of dopamine and glutamate signaling, may be an ideal target for reversing striatal signaling changes caused by chronic cocaine. These effects also verify that at least two separate populations of adenosine receptors with opposing effects on cocaine-

mediated behaviors exist within the striatum. Promoting activation of indirect pathway neurons through postsynaptic adenosine A_{2A} receptor stimulation decreases cocaine seeking, while blockade of these same receptors enhances cocaine seeking. Blockade of presynaptic adenosine A_{2A} receptors, on the other hand, decreases activation of the direct pathway by inhibiting glutamate release preventing cocaine seeking. Perhaps most interesting of the findings presented here is that blockade of presynaptic adenosine A_{2A} or A_1 receptors during extinction training can prevent subsequent reinstatement, and is likely the most feasible as a treatment option for addicts. While the experiments presented in this dissertation explore the effects of adenosine receptors on cocaine seeking the identification of the mechanisms responsible for these effects remain elusive and future studies should to explore viable mechanisms underlying the observations presented here.

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